EVALUATION OF WATER LABORATORIES

Recommended by the United States Public Health Service

U. S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
Public Health Service

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Public Health Service
Bureau of State Services (Environmental Health)
Division of Environmental Engineering and Food Protection
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The ENVIRONMENTAL HEALTH SERIES of reports was established to report the results of scientific and engineering studies of man's environment: The community, whether urban, suburban, or rural, where he lives, works, and plays; the air, water, and earth he uses and re-uses; and the wastes he produces and must dispose of in a way that preserves these natural resources. This SERIES of reports provides for professional users a central source of information on the intramural research activities of Divisions and Centers within the Public Health Service, and on their cooperative activities with state and local agencies, research institutions, and industrial organizations. The general subject area of each report is indicated by the two letters that appear in the publication number; the indicators are

EE — Environmental Engineering
AP — Air Pollution
AH — Arctic Health
FP — Food Protection
OH — Occupational Health
RH — Radiological Health
WP — Water Supply and Pollution Control

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PREFACE

Since 1914 the Public Health Service Drinking Water Standards, as promulgated in the Interstate Quarantine Regulations, have served as criteria for evaluating the quality and safety of interstate carrier water supplies. A major portion of these Standards relate not only to bacteriological quality but also to laboratory methods and technical competency of laboratory personnel.

Section 3.13 of the Standards states that bacteriological results may be accepted from the laboratories of the Reporting Agency, usually the State Health Department, local government laboratories, water works authorities, and commercial laboratories, but only when these laboratories have been approved for use by the Reporting Agency and the Public Health Service. Traditionally, the Public Health Service has approved the State laboratories, which in turn, through qualified State laboratory survey officers, certify the laboratories of local health departments, water works authorities, and commercial establishments.

In 1943 Bacteriologist L. A. Black of the Public Health Service developed a Survey Form for Water Laboratories based on the 8th edition of APHA Standard Methods of Water Analysis. These forms were used in the evaluation of Federal, State, municipal, and other laboratories engaged in the bacteriological examination of water. In subsequent years these survey forms have been revised with each new edition of Standard Methods. The forms have been utilized by laboratory personnel of the Public Health Service in periodic evaluations of state laboratories and by laboratory personnel of many States in evaluations of their local water laboratories. Many of these State water laboratory survey officials also have utilized a copy of keyed "Recommendations" developed by PHS laboratory survey officials for their own use in preparing "Narrative Reports" of water laboratory evaluations to accompany the completed survey forms.

These laboratory evaluations have stimulated and encouraged both State and local authorities to improve procedures and have

resulted in greater conformity and accuracy of microbiological analysis. This publication provides performance standards for evaluation of the bacteriological laboratory procedures and equipment as required by the 1962 Edition of the Public Health Service Drinking Water Standards and is in conformance with the 12th Edition of Standard Methods for the Examination of Water and Wasterwater. To promote greater uniformity, the State laboratory survey officers should make every effort to obtain strict and complete adherence to standard requirements and to apply uniformly the recommended procedures in the Drinking Water Standards for decertification when there is failure to correct violations within a reasonable period of time.

Acknowledgments. Credit for the preparation of this manual is due to Mr. Edwin E. Geldreich, M.S., and Mr. Harold F. Clark, M.S. Mr. Clark, prior to his death in 1965, and Mr. Geldreich have for many years prepared notes concerning deficiencies observed during evaluations of laboratories that are responsible for the examination of water supplies. Many of their laboratory research developments into methodology have been adopted by Standard Methods for the Examination of Water and Wastewater. This manual combines not only their observations of laboratory deviations from standard procedures but much background material upon which the rationale for these procedures is based. Mr. Geldreich has brought their observations up to date and was responsible for the final preparation of this manual in conformance with the latest edition of Standard Methods.

The Survey Form for Water Laboratories (which follows Appendix II) was revised by Luther A. Black, Ph.D., for a more systematic presentation of items to be reviewed in the evaluation of water laboratories.

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INTRODUCTION TO LABORATORY EVALUATION

The objective of a review for a laboratory performing bacteriological examinations of water samples is to increase the quality of technical procedures so that the greatest possible protection is given to the health of the water consumer. It is better for the consultant to consider the review as a conference on methods and procedures as well as to promote the ideas of: cooperation, "Standard Methods" procedures as a protection against criticism of data, advantages of laboratory approval, and an increased knowledge in the field of water bacteriology. This attitude yields much better results with the majority of the laboratories than does having the consultant emphasize the regulatory activities of his visit.

All drinking water and water supply systems used by carriers and others subject to Federal quarantine regulations must conform with provisions of the current edition of "Standard Methods for the Examination of Water and Wastewater (12th edition — 1965)" and "Title 42. Pt. 72 — Interstate Quarantine, Sub-part J — Drinking Water Standards" as amended on page 2152, Federal Register, dated March 6, 1962. These two standards are generally referred to as "Standard Methods" and "Quarantine Regulations" in the remainder of this discussion. The Division of Environmental Engineering and Food Protection of the Public Health Service is responsible for the application of these Standards to interstate carrier water supplies.

Review and Conference on Bacteriological Methods Used for Water in Outline Form

1. Objectives of an Evaluation of a Water Laboratory
   A. Evaluation of official laboratories responsible for bacteriological tests, procedures, and methods of large political subdivisions or area services.
   B. Conference on methods and equipment on request by and for the benefit of the individual laboratory.
   C. Certification of personnel, equipment, and procedures used to examine samples as required under the Public Health Service Drinking Water Standards.
   D. Review of equipment, space, layout, and procedures proposed for a new laboratory to be built or for an existing laboratory to be remodeled.
   E. Attempts to discover causes of discrepancies in results from two or more laboratories performing bacteriological analyses of "split samples" or groups of samples from the same water source.
   F. Investigation of "problem waters" containing atypical bacterial flora or other substances that interfere with routine bacterial examinations.
   G. Examination of a water program and its methods of classifying or assigning supervision and control in an environmental health organization and, administrative responsibility.

2. Local Reasons for a Laboratory Evaluation
   A. Most common requests originate as the result of the desire of an "official laboratory" for a current review of procedures.
   B. Majority of remaining requests originate as a result of the desire of laboratory personnel for a review of their methods or problems or for certification of laboratory services to Public Health Service; or as a result of the need of aid in building a new laboratory, aid in remodeling an existing laboratory, or information on specifications for new equipment.
   C. Infrequent requests for reviews originate as a result of

discrepancies in results from different laboratories or the need for suggestions for investigation of problem waters.

3. Procedure for Requesting Laboratory Review
   A. Request usually originates on local level.
   B. Written request transmitted through supervisory channels to the State laboratory director.
   C. Requests should be made 3 to 6 months in advance of service needs, when possible.
   D. Dates for evaluation visits by State water laboratory survey officials usually are grouped by geographical areas except in an emergency.

4. Preparation for a Laboratory Evaluation

The laboratory evaluation is a scheduled visit. Its basic purpose, regardless of the reason for the requested service, is to benefit the laboratory. In order to secure the maximum good from such a conference, both the consultant and the laboratory staff must cooperate by assuming certain responsibilities in the proposed evaluation.

5. Responsibilities of Consultant in a Laboratory Evaluation
   A. Consider each procedure regarding:
      (1) Choice of method best adapted to local need and coverage.
      (2) Compliance with "Standard Methods" procedures or other acceptable laboratory practices.
      (3) Frequency of examinations, observation of results, and adequacy of data recording for type of sample or source.
      (4) Whether handling and transmission of samples to laboratory follow approved methods or best plan available in local areas.
   B. Examine records and reporting procedures used in laboratory.
   C. Demonstrate technical procedures as needed.
   D. Review equipment, materials, space, and utilities for adequacy of sanitation program of laboratory.
   E. Discuss any deviation from "Standard Methods" procedure or good laboratory practice with personnel con-

cerned. Include an explanation of adverse effects on data.

F. Explain in detail and demonstrate as needed recommended changes for compliance with "Standard Methods." Include justification and benefit to the laboratory and sanitation services.

G. Recommend economies in procedures, time, or materials
H. Invite discussion on methods and results.
I. Discuss problems and proposed investigations.

6. Responsibilities of Laboratory Staff in a Laboratory Evaluation.
   A. Schedule routine procedures of each type of test during the visit of the consultant.
   B. Have tests ready for reading and recording of data if time scheduled for visit is restricted.
   C. Use regular personnel and procedure for the examination of samples.
   D. Have records and work sheets available for the past year.
   E. Prepare summary of number of procedures for each test by months for past year.
   F. Be prepared to discuss difficulties or problems that exist.
   G. Decide how the information and experience of the consultant can best aid the individual laboratory.
   H. Discuss freely the problems and tasks of water bacteriology.

REVIEWING THE LABORATORY WITH THE SURVEY FORM

The form PHS-875 (Rev. 1-66) titled "Survey Form for Water Laboratories (Bacteriological)" is based on the provisions of "Standard Methods for the Examination of Water and Wastewater" (12th edition — 1965). Copies of this form are free upon request to PHS Regional Offices listed on the inside front cover.

An original copy of the survey form should be filled out as the survey is being made in the laboratory. Each item should be reviewed. The marking code consists of an "X" for deviation, an "O" for an item that does not apply to the laboratory being reviewed, and a "U" for items not determined. These marks should be placed in the space provided on the right side of column. On the original survey form, compliance is indicated by a check mark on the left side to indicate to the consultant that the item has been covered, since it is frequently more convenient not to follow the items in numerical order. All information requested should be written on the form in the space provided, as for example, item 48 requests the number of tests per year and percentage of Completed Tests for coliforms in water samples.

Each deviation should be discussed with the staff at the time it is observed. This discussion should include the deviation, effect on validity of results, remedial action, and reasons justifying the change in procedures. Also, essential omissions in procedures are marked with an "X" since the mark "O" is reserved for items that do not apply to the laboratory (see marking code described above).

All interested personnel, including supervisors, should have an opportunity to look over the completed survey form, and each deviation should be discussed by the consultant with the staff. At the same time comments should be invited from the staff concerning the survey.

Finally, a narrative report should be prepared promptly upon the return of the reviewing consultant to his station. This report should include only items discussed with personnel at the time of the laboratory conference.

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PREPARING OFFICIAL COPIES OF THE SURVEY FORMS

Sufficient copies of the survey form PHS-875 (Rev. 1-66) should be prepared for all persons authorized to receive them. Two copies for any survey of laboratories supplying results used in evaluating interstate carrier water supplies should be sent to the PHS Regional Office. The number of copies will depend on the type of laboratory that has been reviewed (State, regional, armed services, municipal water plant, city health, etc.). On the official copies of the survey form, all deviations should be marked (X) or (O), but items of compliance should be left blank. (When typing copies for transmittal to others, do not include check marks at left of items made on original copy.)

Outline for Narrative Report

The narrative report should be prepared according to the following outline and attached to each copy of the survey form:

Report of a Survey of the

(name of laboratory)

at (street address)

(city and state)

on (date)

by

(name, title, organization,

and address

of reviewing consultant)

The equipment and procedures employed in the bacteriological analyses of water by this laboratory conformed with the pro-

visions of Standard Methods for the Examination of Water and Wastewater (12th edition — 1965) and with the provisions of the Public Health Service Drinking Water Standards, except for the items marked with a cross "X" on the accompanying form PHS-875 (Rev. 1-66). Items marked with a "U" could not be determined at the time of the survey. Items marked "O" do not apply to the procedures programmed in this laboratory. Specific deviations are described with appropriate remedial action for compliance in the following recommendations:

Recommendations

List each deviation by item number used on survey form; describe exact deviation; and recommend procedural change for compliance with standard procedure.

Remarks

Comments on procedures, description of unusual tests, or other comments may be included under this center head. If there are no remarks, delete center head from narrative report.

Commendation

If there are items or persons deserving special commendation, place such remarks under this center head. If no commendation is necessary, delete the center head from narrative report. Such commendation should only be used for outstanding performance.

Personnel Approved

Name and title of personnel with the individual tests for which approval is given. Example:

J. Jones, Bacteriologist, Confirmed Test, Completed Test, Plate Count.

J. Smith, Laboratory Aid, Confirmed Test, Direct Coliform (MF) Count.

Names of personnel can be listed by rank, or if the rank is not known, then by alphabetical order.

Conclusion

Give descriptive conclusion, including recommendation for approval or rejection of results of the laboratory. Typical con-

clusions are shown in the following section and, except in special circumstances, one of the conclusions should apply to any laboratory.

.....

Signed by consultant

Conclusions

Conclusion A represents unqualified acceptance and recommended approval of the laboratory and is the highest rating it is possible to give.

Conclusion B is a qualified acceptance with recommended approval where the reviewer believes the deviations do not seriously affect the validity of results and the deviations will be corrected immediately. This conclusion can be made more restrictive, when necessary, by changing the wording following the "it is recommended" to "provisional acceptance of results, pending correction of deviations" or other appropriate wording.

Conclusion C is used where the laboratory, because of lack of equipment or inadequately trained staff or other causes, yields unacceptable results.

Specific conclusions are:

A. The procedures and equipment in use at the time of this survey were in substantial compliance with the provisions of Standard Methods for the Examination of Water and Wastewater (12th edition — 1965) and the Public Health Service Drinking Water Standards, and it is recommended that the results be accepted for the bacterial examination of waters under interstate regulation.

B. The procedures and equipment in use at the time of the survey complied in general with the provisions of Standard Methods for the Examination of Water and Wastewater (12th edition — 1965) and the Public Health Service Drinking Water Standards, and with correction of deviations listed, it is recommended that the results be accepted for the bacterial examination of waters under interstate regulation.

C. The procedures and equipment in use at the time of this survey showed many deviations from the provisions of Standard

Methods for the Examination of Water and Wastewater (12th edition — 1965) and the Public Health Service Drinking Water Standards. It is recommended that results from this laboratory should *not* be accepted for the bacterial examination of waters under interstate regulation. It is further suggested that, after the listed deviations have been corrected, this laboratory request another survey.

KEY TO STANDARD RECOMMENDATIONS

The following keyed "recommendations" for typical deviations in laboratory procedures plus recommended changes are presented only as guidelines in report writing. Mechanical use of these "set" statements on procedures should be avoided whenever possible, since there generally are certain individual characteristics peculiar to a given laboratory. It is better to develop the description of the deviation as it applies specifically to the laboratory being reviewed. However, this set of examples of recommendations could be useful to the new consultant in this review technique and to others of allied professions concerned with public health laboratory activities.

Numbers used in the key follow the same item numbers found on the Survey Form for Water Laboratories (Bacteriological) (PHS-875, Rev. 1-66). Sections under item numbers are listed by letter and contain subordinate information or alternative suggestions for certain specific deviations in a general subject area. Figures enclosed in parentheses indicate page references to "Standard Methods" (12th edition — 1965).

SAMPLES

Item 1. **Sampling**

A. Based on the population served, the number of samples examined each month is approximately .... percent of the minimum number recommended in the Public Health Service Drinking Water Standards, 1962 (page 569-570).

B. Although the number of bacterial samples examined each month comply with existing requirements, it is suggested that the number of samples examined each month be increased to a total of ..... samples per month (page 569-570). This recommendation is based on the belief that sources serving small populations

should examine a greater number of samples (used where less than 15 samples per month are examined).

C. The sample points should be located throughout the distribution system, including representative dead-end mains, to establish adequate bacteriological quality and to demonstrate that no contamination occurs through breaks in the distribution system (page 570).

Item 2. **Collection procedure**

A. It is recommended that a water sample of .... ml should be collected for the bacteriological examination (1.4: page 590).

The minimum standard test portion of 10 ml should be examined with not less than 5 portions each from a single sample (or alternatively 5 portions of 100 ml each) for a single standard test [Title 42. Pt. 72 — Interstate Quarantine, Drinking Water Standards, 72.201 hl (i) and (ii)].

B. It is not permissible to examine single portions from samples collected at different times and add the portions to make a single standard sample.

C. Steps in sampling are completed in the following order:

1. *Select the sampling fixture.*

   A tap, such as a faucet, petcock, or small valve, without leak at the bonnet or stem, is preferable. A large valve or nipple is a second choice because of the greater difficulty in sterilizing and the lesser control over the issuing stream. Fire hydrants are poor sampling stations because of the impossibility of sterilizing the long barrel; in such cases, reliance is placed on lengthy flushing. Bacteria find a favorable food supply in the leather parts of the valve and may wash off into the passing water. After rains, an empty fire plug may readily become contaminated through the weep hole.

2. *Sterilize the outlet.*

   Sterilize the nozzle by flaming with an alcohol lamp, blow torch, or torch made by tightly twisting a sheet of paper. A cotton wad soaked in alcohol and held by a twist on the end of a wire is a handy torch.

   Precaution should be observed to prevent contamination of the sample at the time of collection (add here any recommendation necessary to correct poor sample collection technique) (1.3: page 589).

3. *Flush out the line.*

Draw water for 3 or 4 minutes or until it is judged that contents of the line have been completely displaced back to the main, or until the water shows normal turbidity. When sampling from a deep well, all drop pipe water should be changed.

Tap should be allowed to waste water until the collector is assured that the sample taken for the bacterial analysis has not been standing in a stagnant service line (1.3:page 589).

4. *Have the sampling bottle ready.*

Unwrap the sterilized bottle and loosen the paper covering the cap. Do not touch the mouth of the bottle or stopper with fingers, otherwise the sample will show contamination. Handling only the bottom tip of the paper, spread it out like an umbrella over the glass stopper or screw cap.

5. *Catch the water sample.*

Remove the stopper, holding the bottle by the paper over the mushroom top, and almost fill the bottle in the stream of water from the tap.

Samples from open reservoirs should be scooped up from about a 1-foot depth by a quick pass while the bottle is held mouth forward. This is to prevent water in contact with the hand from flowing into the bottle. Avoid locations where scum or dust floats on the surface.

An ample air space should be left in each sample bottle at the time of collecting the sample in order that it may be adequately mixed at time samples are removed for bacterial examination (1.3:page 589). Particulates that may have adsorbed bacteria on their surfaces settle out during standing, and resuspension of bacterial population is difficult without an air space left for adequate mixing.

The present practice of using sample bottles of 60-ml (2-oz) capacity makes it difficult to obtain both an adequate air space and sufficient water volume for examination. Using larger bottles would necessitate a slight increase in mailing cost for individual samples, but would ensure a more representative examination.

6. *Replace the stopper.*

Replace the cap and press the paper into position, again without accidentally contaminating the mouth of the bottle with the fingers.

7. *Label the bottle.*

The label should state plainly the source of the supply, the exact location sampled, the chlorine residual found at the time of sampling, and the time of day. Note any special conditions that may suggest contamination, so that in the laboratory proper dilutions may be made to cover the range of expected bacterial concentration. Information on the label may also be an important aid in the interpretation of results.

The identification of the water sample is incomplete because ..... (1.5: page 590).

Item 3. **Dechlorination**

A. All samples of water collected from chlorinated sources must be dechlorinated *at time of collection* since failure to do so may result in bacterial death before the bacterial analysis is started, which would result in an underestimation of contamination. The final concentration of sodium thiosulfate, 100 mg/l, is reached by adding an appropriate quantity in solution to the sample bottle before sterilization (1.2: page 588).

B. The quantity of sodium thiosulfate in the water sample bottle should be changed from ..... mg to ..... mg (equivalent to 100 mg/l) (1.2: page 588).

Item 4. **Temperature and storage**

A. The sample should be maintained at a temperature as close as possible to the water temperature at the time of collection until it can be examined (2: page 591). (Note: Do not emphasize this item since it may be extremely difficult to comply with the provisions of "S.M." on storage temperature.)

B. It is suggested that samples collected in the water plant or the laboratory should be examined within an hour or less after collection (2: page 591).

C. Samples collected in the field must be examined within 30 hours after collection or discarded. If the elapsed time is greater than 30 hours, bacterial flora may undergo marked changes.

D. The current practice of accepting 2- to 4-day-old samples for examination should be discontinued. In some instances, the samples could be received and examined within 24 hours if they were collected the first part of the week and promptly mailed.

E. Laboratory records indicate that mail service is adequate for ensuring 30-hour delivery when attention is given to transportation schedules.

F. If samples cannot be examined within 30 hours after collection, alternative provisions should be made to have the sample examined at the site of collection or by an acceptable private or public laboratory closer to the area.

Item 5. **Remedial action for unsatisfactory sample**

A. After a single unsatisfactory sample is examined, it is necessary to examine samples from the same point at daily intervals until two consecutive negative samples are secured (570).

Item 6. **Record of laboratory examinations**

A. The laboratory or engineering section should have available for inspection all records and work sheets on water examinations for the past 6 months.

B. Reports should be prepared promptly on all laboratory results and submitted to the proper authorities. Unsatisfactory results should be immediately followed with a request for another sampling. The frequency of sampling public water supplies located in various areas of the state must be monitored from the laboratory or engineering files. Notice must be sent immediately to those water plant operators who are slow to submit samples.

C. An annual review of laboratory procedures should be performed at each of the municipal or commercial laboratories authorized for public water analysis by the State health department. Such laboratories must meet the approval of the State-appointed consultant. When a laboratory has deficiencies under the Public Health Service Drinking Water Standards or under State regulations because of many deviations, a repeat evaluation should be performed after approximately 3 months to verify that listed deviations have been corrected.

LABORATORY APPARATUS

Item 7. **Sample bottles**

A. It is recommended that soft glass sample bottles be replaced with borosilicate or other non-corrosive glass or plastic sample

bottles that can be sterilized, since soft glass adds to some water samples substances that increase growth during short-time storage of some coliform bacteria and may result in changes in pH (14: page 574).

B. The size of the sample bottle should be increased to 120 ml (4 oz) so that adequate water samples can be collected and leave air space in the bottles to permit proper mixing of sample before bacteriological analysis is started (14: page 574).

C. Glass-stoppered bottles should be covered, before sterilization, with a metal foil, rubberized cloth, or impermeable paper caps to prevent contamination of bottle mouths after sterilization (14: page 574).

D. Screw-cap closures do not require a cover, and to simplify work, the use of covers may be discontinued.

E. New plastic screw caps on sample bottles should be checked for bacterial toxicity. Ten sterile plastic caps should be soaked in 100 ml sterile distilled water for 24 hours at room temperature. A suitability test should be performed on the distilled water (rinse water) used to soak these plastic caps. Those caps that are demonstrated to have toxic residuals, as reflected by the results of the rinse water examination, should not be used until free of these toxic properties. Some new screw caps have been demonstrated to have a phenol residual that requires six successive autoclavings in repeated changes of distilled water before the toxic properties are removed.

F. Wide-mouth sample bottles are preferred because they permit the sample to be taken with less chance of accidental contamination at the water tap. Laboratory technicians also find wide-mouth bottles easier to handle when inoculating presumptive medium with a 10-ml pipet.

Item 8. **Thermometers**

A. The accuracy of all laboratory thermometers used to measure temperatures in water samples, water baths, hot-air sterilizing ovens, and autoclaves should be verified. Verification of accuracy should be made at selected temperatures within the minimum and maximum range of intended use by comparison with the readings on a thermometer certified by the National Bureau of Standards or one of equivalent accuracy. Thermometers should be rechecked periodically to ensure their accuracy.

B. The mercury column in the thermometer must not be

broken by air space separations. This results in loss of measuring accuracy.

Item 9. **Incubator**

A. Temperature variations in the incubator should be determined by placing accurate thermometers at several points on the shelves with the incubator filled (1:page 571).

B. If there is not sufficient incubator space for bacteriological work during peak loads, the purchase of a new incubator with at least double the incubation space is recommended (1:page 571).

C. It is recommended that thermometer bulbs in the incubator be continuously immersed in liquid (glycerine, water, or mineral oil) and a daily record of readings maintained (1:page 571).

D. A daily record of incubator temperatures is required by Standard Methods in the absence of a recording thermometer (1:page 571). This record should include the date and temperature and the initials of the person who recorded the data. Any deviations greater than ± 0.5°C from the 35°C incubator temperature should be corrected by proper thermostat adjustment. Maintaining such a record of incubator temperature will also alert laboratory personnel to any gradual changes that may reflect possible metal fatigue in the bi-metallic strip of the thermostat.

Item 10. **Incubator room**

A. The incubator room is evaluated according to whether it meets requirements for the incubator described above in Item 9 (1:page 571).

Item 11. **Hot-air sterilizing oven**

A. The oven should be checked to ascertain that a uniform temperature is obtained on all shelves at 160° or 170°C where glassware is sterilized by the hot-air technique (2:page 572).

B. The size of the oven is not sufficient for the quantity of glassware to be sterilized. It is recommended that the oven be replaced by one of approximately ..... x ..... x ..... inches (inside measurement).

C. Replacement of the sterilizing oven is recommended if it does not maintain a sterilizing temperature of 170°C because of poor insulation and heating capacity.

D. The oven should be equipped with a temperature-measuring device that is accurate in the 160° to 180°C temperature range (2:page 572).

Item 12. **Autoclaves**

A. The chamber of the autoclave should be equipped with an accurate thermometer with bulb properly located in the exhaust line so that it registers the minimum temperature in the sterilizing chamber (3:page 572).

B. If the autoclave frequently heats slowly and often fails to reach sterilizing temperature in the maximum allowable time of 30 minutes, probably the gas heating unit is poorly adjusted or improper type jets are used for the composition of the available gas (3:page 572).

C. Because of low gas pressure, a high-pressure steam line should be provided or the present autoclaves replaced to ensure that lactose broth or media containing lactose will not be exposed to any heat longer than 60 minutes during sterilization of media.

D. Only the thermometer should be used for recording temperature in the autoclave, since the pressure gage, in relation to temperature, is influenced by numerous variables, such as incomplete exhaustion of all the air in the chamber (3:page 572).

Item 13. **Colony counter**

A. An approved counting aid should be provided that will give uniform and properly controlled illumination (equivalent to that provided by the Quebec Colony Counter) at a magnification of about 1½ diameters on a non-glaring ruled guide plate (rulings in squares preferred) (5:page 572). Note: This recommendation is used only where routine plate counts are a part of the control procedure.

Item 14. **pH equipment**

A. It is recommended that an accurate electrometric pH meter be obtained (6:page 572). Colorimetric methods are not acceptable because it is impossible to make some of the required measurements, such as pH on brilliant green lactose bile broth.

B. Accurate buffer standards should be provided for checking the accuracy of pH test equipment.

Item 15. **Balances**

A. A balance with a sensitivity of 2 grams at a 150-gram load should be obtained for the laboratory (7:page 573).

B. The weights for the balance should be replaced with weights of good quality to ensure weighings of reasonable accuracy.

C. An analytical balance with a sensitivity of 1 mg or less at a 10-gram load should be used for weighing quantities of 2 grams or less (7:page 573).

Item 16. **Media preparation utensils**

A. Pyrex glass or other suitable noncorrosive utensils, such as stainless steel *or complete enamel surface,* are recommended for use in the preparation of media. Utensils that could contaminate the media with copper, zinc, aluminum, etc., should not be used (8:page 573).

B. Utensils used in the preparation of media should be chemically cleaned before they are used (8:page 573).

Item 17. **Pipets**

A. A representative portion of each new lot of pipets should be tested to determine accuracy of calibrations (9:page 573).

B. Pipets should conform to A.P.H.A. specifications (see Standard Methods) (9:page 573).

C. Pipets that are graduated to the tip should be discarded if the tip (is broken) (is cut off) since the measurement of the sample will not meet the required tolerances of error ($\pm 2.5\%$ error) (9:page 573).

Item 18. **Pipet containers**

A. Metal boxes are recommended for sterilization and storage of sterile pipets. The metal box may be made of stainless steel or other non-corrosive, heat resistant metal (10:page 573).

B. Paper wrappings of good quality sulfite paper (kraft) may be used for wrapping small tubes or individual pipets for sterilization (10:page 573).

C. Metal boxes of stainless steel resist heat and last longer than those constructed of aluminum although such heat resistant metal may be used; copper cans or boxes should not be used as pipet containers.

D. Pipets should be sterilized, stored, and handled in suitable closed containers (paper allowed) (10:page 573).

Item 19. **Dilution bottles or tubes**

A. Soft glass containers should be replaced with borosilicate or other non-corrosive glass. Plastic bottles constructed of non-toxic materials of acceptable size may be used (11:page 573).

B. Containers used for dilution bottles (or tubes) must have glass stoppers, rubber stoppers, or leak-proof screw caps (11:page 573).

C. It is the responsibility of the laboratory to prove the dilution vessel and closure are free from toxic substances that may be liberated during sterilization (11:page 573).

D. Dilution tubes or bottles should have the graduation mark indelibly placed on the vessel and its accuracy with tolerances (at ± 0.2 ml or ± 2.0 ml) verified (11:page 573). This will assist the bacteriologist in detecting occasional dilution bottles (or tubes) that may contain improper volumes of dilution water in sterile dilution blanks.

Item 20. **Petri dishes**

A. Glass petri dishes 100 mm in diameter and at least 15 mm high are recommended for the standard plate count (12:page 573).

B. Petri dishes with scratched or etched bottoms should be discarded (12:page 573).

C. It is recommended that petri dishes with irregularities (not having flat bottoms) in the bottom be discarded (12:page 573).

D. Plastic petri dishes that are free from toxic substances and meet the specifications for glass dishes may be used (12:page 573).

Item 21. **Petri dish containers**

A. A suitable container for petri dishes should be used to protect these items from accidental breakage and dust contamination in storage, and for packaging convenience for hot-air sterilization.

B. Stainless steel or aluminum cans with covers are preferred. However, petri dishes may be wrapped in good quality sulfite paper (kraft) or aluminum or lead foil.

Item 22. **Fermentation tubes**

A. The tube containing the medium and inverted vial should be of sufficient size so that the medium plus sample does not fill the tube more than half full (13:page 574). Tubes containing an excess of fluid are subject to contamination.

B. The size of the inverted vial (fermentation tube) is critical, with reference to the medium tube size and the amount of medium. The volume of medium should be sufficient to ensure filling the fermentation vial during sterilization and leaving an equal volume or more in excess so that this vial will partially submerge. In most laboratories, 10 ml of sterile broth is used in each tube since the inverted vial is 75 mm long and has a 4-ml capacity. Smaller inverted vials might be used, but they probably would have insufficient size for reading gas production.

Item 23. **Cleaning glassware**

A. Suitable indicators or pH tests with electrometric equipment should be used to ensure that no alkaline or acid material is a residue on washed and dried glassware.

B. It is the responsibility of the bacteriologist to demonstrate that washed glassware is free of toxic residues or inhibitory residues from the washing procedure (1:page 574).

Test for Detergent Suitability

1. Wash and rinse some petri dishes in usual manner.
2. Rinse another group in six successive rinses of distilled water.
3. Wash some with detergent wash water (in-use concentration) and drain dry without rinsing.
4. Sterilize all three groups.
5. Pour duplicate or triplicate plates with same water sample (20-60 colonies per plate) on dishes cleaned as in 1, 2, and 3, and incubate as usual.
6. If plates not rinsed (3) show a lowered count (15% or more), the detergent contains bacteriostatic substances and another detergent should be selected.
7. If plates in group 1 show a lowered count (15% or more), toxic residues are left on glassware during routine washing procedure and a nontoxic detergent must be substituted for the one in use.

PREPARATION

Item 24. Sterilization of glassware

A. Glassware not in metal containers must be sterilized by the hot-air procedure for not less than 1 hour at 170°C, whereas glassware in metal containers must be heated for 2 hours at 170°C to ensure heat penetration and sterilization (page 575). Hot-air sterilization for 2 hours or longer at 170°C is permitted for all glassware unless some material is charred or decomposed. (Note: the exception to this rule is that a temperature of 160°C may be used where recording temperature devices of known accuracy are attached and in use for oven temperature — page 575).

B. Where the autoclave is used for the sterilization of sample bottles other than of plastic, the temperature of the steam under pressure must be 121°C and maintained for 15 minutes. Pressure measurement is not an acceptable substitute for a temperature reading (page 575).

C. Plastic sample bottles that distort on autoclaving may be sterilized by use of ethylene oxide gas.

Ethylene oxide has been used at times to sterilize heat sensitive materials. This compound is a gas at room temperature and is extremely flammable. As little as 3 per cent in air will support combustion and will explode if confined. When ethylene oxide (usually 11 percent) is mixed in the proper proportions with Freon 11 and Freon 12, it is non-flammable and may be safely mixed with air in any proportion. Cryoxcide (American Sterilizer Company, Erie, Pennsylvania) and Steroxcide (Wilmot Castle Company, Rochester, New York) and two formulations of this type and are available in pressurized aerosol-type cans for convenient use. Because they are in gaseous form, they must be used in a pressure-tight vessel such as a converted autoclave.

The bactericidal effects of ethylene oxide are dependent upon temperature and, to a smaller extent, upon humidity. At 25°C and 40 percent relative humidity, ethylene oxide at a concentration of 800 mg/l in air will kill the most resistant bacterial spores in 2½ hours, and at 1500 mg/l in 1 hour and 40 minutes.

Although polyethylene is not harmed by ethylene oxide, certain other plastics may be damaged, either by ethylene oxide itself or by the solvent characteristics of the Freon contained.

Item 25. **Buffered dilution water**

A. Tap water (isotonic saline, distilled water) must not be used for dilution water. Only buffered dilution water prepared by adding 1.25 ml of stock phosphate buffer to 1000 ml distilled water and then sterilized may be used (1:page 581).

B. A fresh stock buffer solution should be prepared if the turbidity indicates biological growth in the stock solution. The stock buffer can be stored and preserved at 3° to 10°C.

MATERIALS

Item 26. **Water**

A. Distilled water for microbiological use should be free of inorganic and organic substances, either toxic or nutritive, that could influence survival or growth of bacteria and viruses. For most laboratories, the distilled water supply should be free of microbiological organisms that might contribute inhibitory substances; and in laboratories producing various biologicals, the distilled water must be pyrogen-free (page 578).

B. Many factors can influence the quality of a laboratory water supply: (1) design of the distillation apparatus; (2) source of raw water; (3) condition of the deionizing column, if used; (4) use of a carbon filter; (5) storage chamber for reserve supply; and (6) temperature of stored supply and duration of storage before use. These factors may contribute varying degrees of contamination such as metal ions from the distillation system; ammonium hydroxide, hydrochloric acid, and other fumes from the laboratory; chlorine from the tap water supply; and CO_2 from the air.

The distilled water suitability test can be used to evaluate newly installed water distillation apparatus, repaired distilled water systems, and as a periodic check of existing stills for loss of tin plating over a copper base.

C. One criterion of the quality of distilled water is conductivity. Conductivity measurements can be used to indicate the presence of inorganic ions of metals, salts, and bases. The specific resistance of good quality water should exceed 578,000 ohms at 18°C (equivalent to conductivity of 0.5 ppm as NaCl). The conductivity measurement does not, however, distinguish between the presence of toxic or nontoxic metallic ions and does not reveal any organic contaminants that also may be present.

D. When any organic complexes are present in sufficient amounts to be either toxic or nutritive, the first evidence of distilled water impurities may be observed in erratic replicate pour plate or membrane filter counts and irregular growth in certain minimal nutrient culture media. Such difficulties may also be a result of improper washing procedures with detergents that leave toxic residues on glassware items. If the washing procedure and detergent are proven satisfactory, however, then the distilled water supply becomes a prime suspect and should be further investigated.

E. *Aerobacter aerogenes* was chosen as the test organism because it can grow in minimal nutrients and does not require the complex amino acids or other additives that are necessary for *Escherichia coli* or *Streptococcus fecalis*. *Pseudomonas aeruginosa*, in particular, and possibly other pseudomonads will also grow in minimal nutrients, but they would be insensitive to any inhibiting factors from other strains of the same organism present in the unknown distilled water (1.1:page 578).

F. The minimal requirements for a medium to support a moderate growth of *A. aerogenes* are carbon source (citrate), nitrogen source (ammonium sulfate), salt mixture (magnesium, calcium, iron, and sodium), and a buffer (phosphate) solution to keep the pH from shifting into a lethal range. All chemicals used in preparation of these stock solutions should be analytical reagent (AR) grade. This is particularly important in preparing potassium dihydrogen phosphate (KH_2PO_4) since some brands have significant amounts of chemical impurities.

G. All glassware utilized in this procedure should be borosilicate and must receive a final rinse in freshly redistilled water from a glass still prior to dry heat sterilization. This is necessary because the sensitivity of the test depends upon the cleanness of the sample containers, flasks, tubes, and pipets.

H. All stock solutions must be boiled 1 to 2 minutes to kill vegetative cells. These solutions are then stored in sterilized glass-stoppered bottles at refrigerator temperature (5°C) for a period not exceeding 2 weeks to avoid any significant volatile contamination from the air. The salt mixture stock solution will develop a slight turbidity within 3 to 5 days as ferrous salt converts to the ferric salt. Such solutions with a heavy chemical turbidity should be discarded and a new stock solution prepared. Bacterial contamination may also cause turbidity in the buffer solution and this solution should likewise be discarded.

I. The unknown distilled water sample should also be boiled for 1 minute to kill bacterial contamination. Longer boiling periods should be avoided since this may change the chemical composition of impurities in the unknown sample.

Item 27. **Media ingredients**

A. Most *standard* products yield acceptable results. Recommendations can be made to fit the circumstances, such as comparative results with accepted products, where materials come from unknown origin.

B. In general, dehydrated media products are preferable in that minor variations in chemical composition may occur in the preparation of laboratory media. These are eliminated in dehydrated media, resulting in a more uniform product (C: page 581).

C. The lactose used for bacterial fermentation tests should be pure and free from traces of other fermentable carbohydrates, polyhydric alcohols, glucosides, or other fermentable carbon compounds. The following test is satisfactory to exclude from lactose any foreign fermentable substances that may cause difficulties in the common bacterial test procedures with lactose and establish its suitability for use in culture media.

Presumptive test procedure

1. Add 1 percent lactose to tubes containing 5 ml of a broth free from any fermentable carbon compounds and containing phenol red indicator. Phenol red broth base (Difco B 0092) or (BBL 01-173) are suitable media for the broth base.
2. Prepare control tubes containing no added carbohydrate.
3. Add 0.50 percent glucose to tubes of broth.
4. Sterilize at 121°C for 10 minutes.
5. Cool rapidly on removal from autoclave.
6. Using a straight needle, inoculate a tube of lactose broth and control tubes containing (1) glucose broth and (2) carbohydrate-free broth with *Salmonella typhi* and duplicate tubes with *Salmonella paratyphi*.
7. Incubate tubes at 35°C for 48 hours.
8. Examine for turbidity (growth) and color changes of indicator at 24 ± 2 hours and 48 ± 3 hours.

Interpretation of results

1. Suitable lactose for bacteriological culture media will show no acid reaction indicated by color change of phenol red indicator (no yellow color change); will have marked turbidity (growth); and will match the carbohydrate-free control broth tube in appearance and color at the 24-hour and 48-hour readings, whereas the glucose tube will show acid production in 24 hours. Absence of turbidity in tubes indicates unsatisfactory growth for test, and failure of glucose control tube to turn yellow indicates an unsatisfactory bacterial stock culture.
2. An unsatisfactory lactose suitability test shows a trace of acid production (yellowish color) with *S. typhi* or *S. paratyphi* within 48 hours and is presumptive evidence that the lactose may not be suitable for culture media or that the test media have been exposed to excessive temperatures during sterilization. If sterilization time and temperature are in question, then a second test procedure is recommended.

Confirmation test procedure

1. Sterilize the phenol red broth before addition of any lactose. Sterilize the lactose solution by filtration; using aseptic technique, add the sterile lactose in required quantity to phenol red broth. Repeat the lactose suitability test.

Interpretation of results

1. An unsatisfactory lactose test shows the lactose is not suitable for bacteriological work. This confirms the presumptive test and the lactose should be rejected.
2. A satisfactory test by the confirmed procedure indicates faults are occurring during preparation of carbohydrate media. Methods of sterilization should be examined since excessive temperature or excessive exposure to recommended temperature may be producing hydrolysis of the lactose. Traces of acids or mineral salts left during faulty washing procedures may result in lactose hydrolysis.

Item 28. **General chemicals**

All chemicals used in the preparation of culture media must be

ACS or analytical reagent (AR) grade. This is particularly important in media preparation since some chemical impurities found in commercial and other lower grades of chemicals can be of sufficient amount to suppress or inhibit bacterial growth.

Item 29. **Dyes**

Dyes may differ in biological activity from lot to lot and from manufacturer to manufacturer. Dye technology indicates the differences in lots of a given dye are related to dye content produced, mixture of dye complexes present, and amount of inert, non-soluble residuals remaining in the product. Therefore, it is recommended that all dyes used in the preparation of culture media be purchased from lots certified by the Biological Stain Commission for bacteriological use.

Item 30. **Media storage**

A. Culture media should be stored in a clean, cool, dark place free from dust particles, contamination, and excessive changes in temperature (1:page 576).

B. The culture media should be protected from strong light. It is especially necessary to protect brilliant green lactose bile broth from strong daylight, sunlight, or fluorescent light since the brilliant green may be decomposed.

C. Media may be stored at low temperatures (2° to 10°C) or at room temperature, but it is more convenient to store at room temperature where excessive evaporation is not a problem.

D. Media stored in the refrigerator should be removed for storage overnight at room temperature, and any tubes showing turbidity or containing air bubbles in the inverted vials must be discarded (1:page 576).

Item 31. **Adjustment of media reaction**

A. The pH of all batches of culture media should be checked after sterilization and the pH of each batch recorded with date and medium lot number. By monitoring final medium pH, a check can be made on possible errors in proportions weighed, excessive sterilization resulting in lactose hydrolysis, and chemical contamination or deterioration of ingredients that might occur during storage after stock packages are opened.

B. Media that have a final pH value after sterilization greater than ± 0.1 of the correct amount should be discarded and the source of the difficulty located. If this problem is not a result of improper medium preparation, prolonged sterilization, or decomposition of the dehydrated commercial medium product, then distilled water pH may be the cause of the difficulty. Such a condition will then necessitate adjustment of the medium pH prior to sterilization so the final pH value will meet the above requirements.

C. The appropriate standard buffer should be used to calibrate the pH meter. Since most bacteriological media used in the water laboratory are near pH 7.0, it is recommended that the standard buffer used to calibrate the pH meter be within pH ± 1.0 of this range. Since meter scales are not linear across their total range, a standard adjustment at pH 9.0 can be 0.4 unit low when used to measure a solution at pH 7.0.

D. In cases where adjustment of the pH of the medium before sterilization is necessary, allowance must be made for reduction of pH (usually 0.1 to 0.2 unit) during autoclaving. The drop in pH will be a characteristic of the individual autoclave (2: page 576).

Item 32. **Sterilization of media**

A. All media, with the exception of sugar broths, should be sterilized at 121°C for 15 minutes, unless there are specific directions for another temperature or time. Broths containing sugars may be sterilized at 121°C for 10 to 15 minutes at the option of the operator (3: page 577).

B. Sterilization temperatures must be determined by an accurate thermometer located in the exhaust line and not by the reading of the pressure gage (3: page 572).

C. The maximum elapsed time for exposure of sugar broths to any heat (from the time the autoclave door is closed until the medium is removed from the autoclave) is 1 hour. Excessive exposure of sugars, especially lactose, may result in hydrolysis, which in turn will give false-positive reactions with some noncoliform bacteria. Therefore, it is recommended this autoclave be repaired or replaced to meet the above 1-hour maximum. This interpretation is somewhat more liberal than the recommendation of 30 minutes in Standard Methods (3: page 582) for the preparation of lactose broth.

Item 33. **Clarification**

Media clarification is not generally necessary when commercial dehydrated products are used. Such preparations generally result in clear media solutions or solutions with only a trace of fine colloidal turbidity.

CULTURE MEDIA — SPECIFICATIONS

Item 34. **Percent solution**

A. Percent solution is defined as grams solute per 100 ml of solution.

B. Freshly distilled water should be used in the preparation of all media.

C. To the dry flask, dry ingredients should be added first. Then, while the flask is agitated constantly, small portions of distilled water should be added. This procedure speeds up the wetting of powder and reduces the formation of lumps.

Item 35. **Nutrient broth**

The formula for nutrient broth (in grams/liter) consists of beef extract (3 g), peptone (5 g), and 1 liter of distilled water. Final pH is 6.8 to 7.0 (2:page 582).

Item 36. **Lactose broth**

A. Formula for lactose broth (in grams/liter) consists of beef extract (3 g), peptone (5 g), lactose (5 g), and 1 liter of distilled water. Final pH is 6.8 to 7.0 (3:page 582).

B. The most common errors involve the concentration of lactose broth.

1. It is recommended that not less than 10 ml of sterile broth be used in each tube.
2. Quantities of ingredients are insufficient for the quantity of water sample added to the lactose broth. Except for volumes of "1 ml or less of water sample" added to 10 ml broth, the concentration of ingredients in the broth plus added water sample must equal normal strength broth. For example, 10 ml of double-strength broth plus 10 ml water sample

equals 20 ml of single-strength broth by dilution, with each milliliter in the planted tube containing the equivalent of 0.013 gram of dehydrated medium (3:page 582). Where irregular volumes are used, the grams dehydrated medium (lactose broth only) needed per liter is calculated as follows:

$$\frac{(X)\ (\text{ml broth})}{\text{T.V.}} = 13.0$$

where X = number of grams per liter in lactose broth

ml broth = number of milliliters broth per sterile tube

T.V. = number milliliters sterile broth plus milliliters of water sample added.

$$X = \frac{(13)\ (\text{T.V.})}{\text{ml sterile broth per tube}}$$

Lactose broth with ..... ml of broth and ..... ml water sample should contain ..... grams dehydrated medium per liter (3:page 582).

Item 37. **Lauryl tryptose broth**

A. Formula for lauryl tryptose broth (in grams/liter) consists of tryptose (20 g), lactose (5 g), dipotassium hydrogen phosphate (2.75 g), potassium dihydrogen phosphate (2.75 g), sodium chloride (5 g), sodium lauryl sulfate (0.1 g), and 1 liter of distilled water. Final pH is 6.8 ± 0.1 (4:page 583).

B. See recommendations in item 36.

C. The quantity of lauryl tryptose broth per liter is calculated with the following formula (X = grams dehydrated medium per liter).

$$\frac{(X)\ (\text{ml broth})}{\text{T.V.}} = 35.6 \quad \text{or} \quad X = \frac{(\text{T.V.})\ (35.6)}{\text{ml LST broth per tube}}$$

D. In laboratories where the percentage of confirmations in brilliant green lactose bile broth is very low compared to the number of positive presumptive tubes in lactose broth, the reviewer is justified in recommending a change from lactose presumptive broth to lauryl tryptose broth. This change usually reduces the number of falsely-positive reactions due to noncoliform bacterial flora. In certain rare types of surface water,

the change may not result in much benefit in reducing false-positive presumptive tests.

Item 38. **Brilliant green lactose bile broth**

A. Formula for brilliant green lactose bile broth (in grams/liter) consists of peptone (10 g), lactose (10 g), dehydrated oxgall (20 g), brilliant green (0.0133 g), and 1 liter of distilled water. Final pH is 7.0 to 7.5 (9:page 584).

B. Excessive exposure of media to heat, especially those media containing a carbohydrate, may produce hydrolysis (as lactose to glucose and galactose) of the carbohydrate, resulting in gas production by lactose negative, glucose positive microorganisms with false-positive results.

C. Colorimetric methods are not applicable for the determination of the pH of strongly colored solutions, brilliant green lactose bile broth for example. The pH of colored solutions is determined by the electrometric method.

Item 39. **Endo medium**

A. Formula for Endo agar (in grams/liter) consists of peptone (10 g), lactose (10 g), dipotassium phosphate (3.5 g), sodium sulfite (2.5 g), basic fuchsin (0.4 g), agar (20 g), and 1 liter of distilled water. Final pH is 7.4 ± 0.1 (7.2:page 584).

B. Endo agar is best prepared by combining the peptone, buffer, lactose, and agar into a stock agar, which is boiled to dissolve the agar or sterilized in an autoclave for 10 minutes at 121°C. To stock agar cooled to 44° to 45°C, the indicator system is added (basic fuchsin alcoholic solution and sodium sulfite). After mixing, plates are poured with the usual precautions against contamination and allowed to harden.

C. Sterilization of a complete Endo agar (lactose agar base plus indicator system) at 121°C for 15 minutes is not recommended. Excessive heat destroys the sodium sulfite, which results in poor sheen development on coliform colonies.

Item 40. **Eosin methylene blue agar**

A. Formula for eosin methylene blue agar (in grams/liter) consists of peptone (10 g), lactose (10 g), dipotassium phosphate

(2 g), eosin Y (0.4 g), methylene blue (0.065 g), agar (15 g), and 1 liter of distilled water (8:page 584).

B. Eosin methylene blue agar is best prepared by combining the peptone, buffer, lactose, and agar into a stock agar, which is boiled to dissolve the agar or sterilized in an autoclave for 10 minutes at 121°C. To stock agar cooled to 44° to 45°C, the indicator system is added (eosin aqueous solution and methylene blue aqueous solution). After mixing, plates are poured with the usual precautions against contamination and allowed to harden.

C. The E.M.B. agar of Holt-Harris and Teague for isolation of intestinal pathogenic bacteria should not be used in water bacteriology. This medium contains saccharose in addition to lactose, which could result in a significant increase in non-coliform organisms appearing as coliform colonies on this version of E.M.B. agar. The use of Levine E.M.B. agar is recommended for confirmation or isolation of coliform bacteria in water bacteriology. (8:page 584).

Item 41. Tryptone glucose beef extract agar and tryptone glucose yeast extract agar

A. Formula for tryptone glucose beef extract agar (in grams/liter) consists of beef extract (3 g), tryptone (5 g), glucose (1 g), agar (15 g), and 1 liter of distilled water. Final pH is 6.8 to 7.0 (5:page 583).

B. Formula for tryptone glucose yeast extract agar (in grams/liter) consists of peptone-tryptone (5 g), yeast extract (2.5 g), glucose (1 g), agar (15 g), and 1 liter of distilled water. Final pH is 7.0 ± 0.1 (6:page 583).

C. Sterile medium should not be remelted a second time after sterilization because insoluble compounds may appear and interfere with interpretation of colony counts.

Item 42. M-Endo (MF) medium

A. Formula for M-Endo (MF) medium (in grams/liter) consists of tryptone or polypeptone (10 g), thiopeptone or thiotone (5 g), casitone or trypticase (5 g), yeast extract (1.5 g), lactose (12.5 g), sodium chloride (5 g), dipotassium hydrogen phosphate (4.375 g), potassium dihydrogen phosphate (1.375), sodium lauryl sulfate (0.050 g), sodium desoxycholate (0.1 g), sodium sulfite (2.1 g), basic fuchsin (1.05 g), and 1 liter of distilled water con-

taining 20 ml of 95 percent ethyl alcohol. Final pH is 7.1 to 7.3 (12:page 585).

B. The medium should be prepared in clean, pre-sterilized borosilicate glassware to reduce any occurrence of chemical or bacterial contamination.

C. The addition of ethyl alcohol (U.S.P. or CP grade) to a final concentration of 2 percent is essential so that the coliform colonies will develop a maximum sheen and have less tendency toward confluence of colonies.

D. Excessive heating of this medium destroys or reduces its specificity, and it is recommended the medium only be heated to the boiling point (but *not boiled*) in its reconstitution (12:page 585).

E. M-Endo medium is sensitive to light and should not be exposed to strong light or sunlight (discard unused medium after 96 hours).

F. The medium used for the MF test has an incorrect formula on comparison to Standard Methods. EHC Endo, M-Endo broth w/B.G., M-HD Endo, M-HD Endo broth w/B.G. are not recommended Endo-type media. This medium should be discarded and one with the correct formula substituted (12:page 585).

Item 43. **LES Endo agar medium**

A. Formula for LES Endo agar (in grams/liter) consists of yeast extract (1.2 g), casitone or trypticase (3.7 g), thiopeptone or thiotone (3.7 g), tryptose (7.5 g), lactose (9.4 g), dipotassium hydrogen phosphate (3.3 g), potassium dihydrogen phosphate (1 g), sodium chloride (3.7 g), sodium desoxycholate (0.1 g), sodium lauryl sulfate (0.05 g), sodium sulfite (1.6 g), basic fuchsin (0.8 g), agar (15 g), and 1 liter of distilled water containing 20 ml of 95 percent ethyl alcohol. Final pH is 7.1 to 7.3 (14:page 585).

B. Excessive heating may destroy this medium or reduce its specificity for coliform differentiation. LES Endo agar should be heated to boiling for a complete solution of the agar and then cooled to 45° to 50°C for preparation of agar plates.

C. Plates of LES Endo agar may be stored for periods up to 2 weeks if kept in the dark at 2° to 10°C. This medium is sensitive to strong light.

MULTIPLE-TUBE TECHNIQUE

Item 44. Presumptive test

A. Both lactose broth and lauryl tryptose broth are permitted as presumptive test media. Where both media give equivalent results on confirmation, it is more economical to use lactose broth. However, where 20 percent or more of the presumptive positive tubes fail to confirm, the use of lauryl tryptose broth should be recommended in place of lactose broth. In many types of water, the lauryl tryptose broth gives a marked reduction in false-positive tubes, resulting not only in savings of labor, brilliant green lactose bile broth, and time, but also in more rapid reporting of negative tests. In all other instances, the choice of presumptive medium should be left to the analyst.

B. The standard sample for the bacteriological coliform test consists of five standard portions of (1) 10 ml each or (2) 100 ml each (Title 42, Pt. 72, Subpart J. Drinking Water Standards, Sec. 72.201 or Standard Methods — 12th edition, page 595. Since the additional samples of 1 ml (1 tube) and 0.1 ml (1 tube) are not required by standards in the potable water test, it is not necessary to use the additional materials, tubes, and labor for the 1-ml and 0.1-ml samples in single strength lactose broth.

C. See items 36 and/or 37 for recommendations on concentration of ingredients in the presumptive tube. The most common error is to use 10 ml or 20 ml of single strength broth for a 10-ml water sample, resulting in half- or two-thirds-strength presumptive medium (final concentration).

Positive tube (presence of gas in *any quantity*) may be confirmed as soon as gas appears. In routine practice, presumptive tubes are examined at 24 ($\pm$ 2) hours and 48 ($\pm$ 3) hours. It is essential that all positive tubes be confirmed at each reading period. The practice of submitting positive tubes to the confirmed test at the end of 48 hours as a substitute for the 24-hour period should not be permitted. Because of the mixed bacterial flora growing with coliform bacteria in the presumptive medium, tubes containing coliforms that produce gas in 24 hours frequently give negative confirmations from the same tube after 48 hours incubation. This attenuation or destruction of these coliforms may be a result of low pH or antagonistic action of other organisms in the heterogenous bacterial flora.

D. If any gas is present in the inverted vial, the test should be recorded as a *positive presumptive test* (1:page 596).

E. It is recommended that records show presence or absence of gas production in the presumptive test after 24 and 48 hours as required by Standard Methods (1:page 596).

F. The presumptive test should be confirmed if gas in *any quantity* is present after the second 24-hour incubation but negative after the first 24-hour incubation period (2.1:page 597).

G. The fermentation tube showing gas should be shaken gently or mixed by rotating before planting confirmatory media are planted (2.3:page 597).

H. Use of a pipet for transfers from positive primary fermentation tubes is no longer permitted in Standard Methods. Use of a 3-mm or larger diameter loop is required (2.3: page 597).

I. Presumptive tubes that fail to show gas in 48 (±3) hours at 35°C (± 0.5°) must be considered negative, even if submitted to the "confirmed test" and the presence of coliform bacteria is demonstrated (1.2:page 596).

The above recommendation is based on the premise that the positive test is due to slow lactose fermenters that are (1) excluded by definition and (2) of unknown sanitary significance.

This premise is obviated if the laboratory technique and practices are not in accordance with Standard Methods, e.g., media of substandard productivity, toxic distilled or sample waters, too high or too low incubation temperatures, etc.

Item 45. **Confirmed test**

A. Procedures permissible in the confirmed test procedure are (1) confirmation in brilliant green lactose bile broth; (2) streaking on eosin methylene blue agar and recognition of typical and atypical colonies; and (3) streaking on Endo agar and recognition of typical and atypical colonies (2:page 596). The *brilliant green lactose bile broth procedure (1) is recommended* because:

1. Coliform colonies may not be recognized by an inexperienced worker.
2. Plate procedure requires more time and skill.
3. Coliform organisms may be lost during the streaking of the plate.

B. The presumptive tube should be shaken gently to ensure mixing before a loop is transferred to the confirmatory tube or plates are streaked for confirmation (2.3a:page 597).

C. Confirm all positive presumptive tubes at end of 24 hours and repeat for subsequent positive presumptive tubes at end of 48 hours. Letting confirmation of 24-hour positive tubes go until 48 hours is not permitted (2.1:page 596). The practice of incubating all presumptive tubes for 48 hours prior to confirming positive tubes in brilliant green lactose bile broth is unsatisfactory. Because of the mixed bacterial flora growing with coliform bacteria in the presumptive medium, tubes containing coliforms that produce gas in 24 hours frequently give negative confirmation from the same tube after 48 hours incubation. This attenuation or destruction of these coliforms may be the result of low pH or antagonistic action of other organisms in the heterogeneous bacterial flora for a given water sample.

D. The presence of gas in any quantity should be recorded as a positive reaction (2.3c:page 597).

E. When solid medium is used in the confirmed test, plates should be streaked so that isolated colonies are obtained. A colony should be read only if it is at least 0.5 cm from another colony (2.4:page 597).

F. Typical colonies that are nucleated with or without sheen should be recorded as positive; opaque, unnucleated pink colonies as doubtful; and only colorless as negative (2.4c:page 597).

G. In the absence of typical colonies and with atypical colonies present, the atypical colony must be examined by the completed test procedure (2.4c:page 597).

Item 46. **Completed test**

A. The "completed test" procedures should be applied only to positive "confirmed test" tubes or plates of solid differential medium (3:page 598).

B. The complete test is recommended where there is doubt as to the validity of the confirmed test.

C. A negative completed test is a negative test for the coliform group and should not be reported as confirmed test-positive and completed test-negative but as non-members of a coliform group demonstrated in sample.

D. Completed test is positive for a coliform group when the pure culture produces gas in lactose broth (or lauryl tryptose broth) and consists of gram-negative, non-spore-producing rods (3.2:page 598).

E. Completed test is doubtful and should be examined further when gas is produced in lactose with a mixture of (1) gram-positive and gram-negative rods or (2) spore-producing micro-organisms and gram-negative rods.

Item 47. Application of tests to routine examination

A. Extreme care should be used in interpreting the data from the "presumptive test" since this procedure tends to give positive reactions with many non-coliform bacterial species.

B. The confirmed test may yield positive reactions in the absence of the coliform group (false-positive test). Therefore, it is necessary to establish the validity of the confirmed test by comparison with the completed test (3:page 601).

C. The number of comparative procedures for establishing the confirmed test for use in water quality examination by comparison with the completed test depends on the individual location. Approximately 20 tests each 3 months should be sufficient where good agreement is secured. The number of tests should be increased if the sanitary interpretation of results from the confirmed test and completed test differ. The completed test is the reference standard.

Item 48. Number of tests per year

A. The laboratory or engineering section should have available a summary of the number of presumptive, confirmed, and completed tests performed by the multiple-tube procedure during the last 12 months.

B. Statistical analysis of the number of tests per year can be used (1) to demonstrate a possible need for lauryl tryptose broth to reduce the frequency of presumptive false-positive results and (2) to test the validity of the confirmed test by sufficient seasonal comparisons with the completed test procedure.

MEMBRANE FILTER TECHNIQUE

Item 49. Application as standard test

Comparison of the membrane filter test and the completed test procedure by parallel testing is mandatory to establish

validity of the MF test. Interpretation of data is based on the membrane filter test procedure and the completed test procedure yielding the same information "relative to sanitary quality of the water supply" (2nd paragraph, page 610): The comparative study is based on sanitary interpretation of the water quality and does not require that the two test procedures demonstrate numerically equal coliform densities.

Item 50. **Laboratory apparatus**

A. See items 7, 17, and 19 for applicable recommendations on sample bottles, pipets, and dilution bottles.

B. Graduated cylinders may be used when sample volume is greater than 20 ml. Most large sample volumes can be measured in 100-ml graduates.

C. It is recommended that separate sterile graduates be used with different samples of especially heavily polluted waters. When this is not possible, the inside of graduate should be rinsed with three small volumes (25-30 ml) of sterile dilution water before the next sample is tested.

D. Containers for culture medium should be of clean borosilicate glass and pre-sterilized to reduce bacterial contamination. Although they may be of any size or shape, Erlenmeyer flasks with metal caps, metal foil covers, or screw caps are recommended for ease of handling at the work bench (1.4:page 611).

E. Although glass petri dishes are recommended for membrane filter tests (1.5:page 611), there are two precautions to consider since such dishes are loose-fitting: (1) prevent possible loss of medium with resultant change in medium concentration and (2) maintain a humid environment for optimum colony development.

F. Most laboratories are using disposable plastic culture dishes, which give a tight fit and thereby reduce the problem of medium evaporation during the 22- to 24-hour incubation period. In such containers, the required humid conditions are established by evaporation of a small portion of the culture medium. There is a negligible change in the culture medium concentration. With the advent of plastic culture dishes, any conventional incubator may be used.

G. Plastic dishes, as supplied by the various manufacturers, are sterile. If reuse of these culture dishes is necessary, then they may be "sterilized" by immersion in 70 percent ethanol for about

30 minutes, air-dried on a sterile towel protected from dust, and reassembled. Such a "sterilization" method may not kill spores, but since these plastic dishes are to be used in coliform recovery, the occasional survival of a spore-forming organism presents no apparent difficulty.

H. Plastic disposable items are generally recommended for one use only. Labor and time involved in preparing them for reuse is usually more costly than the original unit price of such items.

I. Metal ointment boxes are not recommended for culture dishes. Metal plating is not uniform and rust has been found to develop quickly in many of the different manufacturers' lots. Such rust influences growth of the bacterial colonies and should be avoided. This is a particularly undesirable problem when various streptococci media containing sodium azide are used. Sodium azide has a strong metal corrosive action. Another major difficulty is that the plastic coating or lacquer becomes sticky during dry-heat sterilization. Such metal boxes are very difficult to open and must be pried open with a knife or blunt instrument.

J. Filtration assemblies should not leak during filtration. This is frequently caused by worn lock wheels on the funnel-locking ring assembly. Inspection of the narrow neck of the funnel sometimes reveals worn areas through the metal plating with exposure of the brass base. Brass is toxic to bacteria. Newer funnels now being manufactured of stainless steel do not exhibit this problem (1.6: page 611).

K. All surfaces of the filter-holding assembly in contact with the water sample prior to its passage through the membrane filter should be uniformly smooth and free from corrugations, seams, or other surface irregularities that could become lodging places for bacteria.

L. It is recommended that the filter-holding units be polished with a silicone preparation such as "Desicote." This hydrophobic coating prevents metal or glass from being wetted and minimizes sample retention on surfaces of the filter-holding assembly. Silicone coatings on filtration equipment withstand repeated autoclave sterilization. With equipment in daily use, it is suggested that silicone be applied at about 2-month intervals.

Item 51. **Filter membranes**

A. In recent years products of several new manufacturers of membrane filters have appeared on the market. With the in-

crease in competitive selling, there is a growing concern about possible reduction in quality control, which could result in an inferior product being used in bacteriological methodology. The following points should be carefully considered when membrane filters are purchased:

1. The filter should consist of a uniform distribution of pores whose diameter is 0.45 ± 0.02 micron and be free of irregular nonporous areas of 1-micron size or larger. These nonporous areas prevent the diffusion of nutrient through the filter, and any bacterial cells over such areas do not develop into visible colonies. When M-Endo MF medium is used in a test of diffusibility, non-wetting areas on the filter remain white and dry. Such observations should not be confused with air bubbles, which can be removed by reseating the membrane over the medium-saturated pad.
2. The ink used to imprint the grid system on the membrane filter should be non-toxic to any bacteria cultivated on the filter. The ink should not stimulate the development of colony confluency along the grid line system or restrict colony development. Grid line ink should not have a tendency to "bleed" across the membrane after contact with medium during 24 hours of incubation at 44.5°C.
3. The filter disks and absorbent pads should not deteriorate as a result of being sterilized in an autoclave at 121°C (15 psi) for 10 minutes. Disks and pads so deteriorated are not to be used.
4. The membrane filter should provide complete retention of bacterial organisms from liquids and also provide for their suitable cultivation.
5. The membrane filter should not contain any materials that would adversely affect colony growth or interfere with bacteriological indicator systems that are incorporated into culture media for differentiation of organisms.

B. Membrane filter brittleness and distortion of shape may be related to stocks of old membrane filters and sterilization periods in excess of 15 minutes at temperatures higher than 121°C. Purchase of membrane filter stocks should never exceed laboratory needs for a 6- to 12-month period.

C. Membrane filters may be purchased in resealable kraft envelopes containing 10 filters and 10 absorbent pads. These pack-

ages, as sold by the manufacturer, are not sterile, but are packaged *for* autoclaving.

Item 52. **Absorbent pads**

A. Absorbent pads furnished with membrane filters should meet all requirements. The pads may be sterilized with the filters or in separate containers. They must be free of visible moisture prior to use. This eliminates the possibility of the medium being diluted by water condensate still retained after sterilization in an autoclave.

B. Since absorbent pads are used to support bacterial nutrients in the membrane filter procedure, the paper should be of high quality and free of sulfites or other substances that could inhibit bacterial growth. When the quality of the absorbent pads is suspected of contributing to erratic growth of bacteria, the following suitability test is recommended:

1. Soak 10 sterile absorbent pads in 100 ml of distilled water for 24 hours at 35°C.
2. Perform a suitability test on the distilled water (rinse water) used to leach out any soluble substances in the paper.
3. A test lot of absorbent pads demonstrated to have toxic residuals as reflected by the results of the rinse water examination should not be used.

Item 53. **Forceps**

Membrane filters should be handled with sterile forceps on the outer edges, i.e., outside the effective filtering area. This avoids smearing entrapped bacteria or piercing the membrane filter surface, and thus breaking the retention capabilities (1.9: page 613).

Item 54. **Microscope and lamp**

A. Membranes are best counted with a magnification of 10 to 15 diameters with a light source adjusted to give maximum sheen appearance (1.11: page 613). A binocular wide-field dissecting microscope is recommended as the best optical system. However, a small fluorescent lamp with magnifier is acceptable.

B. A microscope lamp consisting of two 4-watt daylight tubes mounted on a flexible arm supported by a heavy cast base is recommended for membrane filter colony illumination.

Item 55. **Filtration**

A. A standard sample of 100 or 200 ml is usually filtered for municipal supplies, i.e., treated water supplies. In this case, this is a procedure to demonstrate less than 1 coliform bacteria per 100 ml, so no attempt is made to establish a lower coliform limit for valid counts.

B. Untreated water supplies believed to be of potable quality may be examined in 50- to 100-ml samples.

C. Water samples that have a high density of particulate material such as clay, colloidal iron, or algae, or that have an extremely high density of non-coliform bacteria with essentially no coliforms present are not suitable for examination by the membrane filter technique. The quantity of water that could be filtered would not be sufficient to measure any coliforms that might be present, since they would be obscured by these particulates. The multiple-tube procedure is recommended for these classes of samples.

D. The amount of medium necessary to saturate the absorbent pad varies as a result of pad thickness and degree of dryness. Any excess of medium is removed from the culture dish prior to rolling the membrane over the absorbent pad. If the excess is not removed, flooding of the membrane will result. This is one cause of confluent growth and contaminated culture medium. Insufficient medium results in small, "starved" colony development.

E. The filter-holding unit should be sterile at the beginning of each filtration series. A filtration series is considered to be interrupted if there is an interval of 30 minutes or longer between sample filtrations. After such interruption any further sample filtration is treated as a new filtration series and requires a sterile filter-holding unit.

F. Membrane filters are extremely delicate and easily damaged. For manipulation, the sterile forceps should always grasp the outer part of the filter disk, outside the part of the filter through which the sample passes.

G. To avoid damage to the membrane filter, the funnel element never should be turned or twisted while being seated and locked to the lower element of the filter-holding unit. The locking ring on the upper element should be turned sufficiently to give a snug fit, but should not be tightened excessively.

H. After all the sample has passed through the membrane

filter, the sides of the funnel walls should be rinsed down with at least 20 ml of sterile dilution water. The rinse should be repeated twice after all the first rinse has passed through the filter. The purpose of the funnel rinse is to flush all droplets of a sample from the funnel walls to the membrane filter. Extensive tests have shown that with proper rinsing technique bacterial retention by the funnel walls is negligible.

I. Air bubbles between the membrane and absorbed pad can easily be recognized on M-Endo (MF) medium as colorless spots in the membrane. A membrane with no air bubbles is uniformly pink. The remedy for bubbles is to lift the membrane with sterile forceps and roll it again onto the absorbent pad.

Items 56 and 57. **MF coliform procedures**

A. The bacteriologist is permitted a choice of methods for detection of coliforms by the membrane filter procedure (5: page 613). Either M-Endo (MF) broth or LES Endo agar may be used. In either case, there is a further choice of enrichment in lauryl tryptose broth or direct culturing on one of these two Endo media.

B. With the several options as to media choice, and with or without enrichment, laboratory personnel have the opportunity to evaluate the methods for best coliform detection in their water supplies. Such an evaluation should include a variety of samples examined during different seasonal periods to find the method giving optimum coliform detection in waters from that geographical area.

C. Many laboratories have found no additional recovery when using an enrichment period. This difference in enrichment requirement is a reflection of the great differences in chemical composition, hydrogen-ion concentration, electrolyte concentration, protein nitrogen, types of bacterial flora present, and perhaps other unknown factors associated with water samples from widely separated geographic areas.

Item 58. **Incubation**

A. The incubator should maintain a high level of humidity (approximately 90 percent). Reduced humidity frequently will permit the surface of the membrane to lose moisture more rapidly than it is replenished by the diffusion of medium from the ab-

sorbent pad. This condition may result in growth failure, or at best, in small or poorly differentiated colonies.

B. A constant-temperature water bath can be modified to make an excellent incubator. The water bath must be covered, and it must have, just above the water level, a suitable platform upon which the culture containers may be placed for incubation. Best results are obtained if a damp towel is placed over the culture containers during incubation.

C. A conventional incubator may be used; however, culture containers must be placed in a tightly closed container, with wet paper or cloth inside to maintain the required humidity conditions. A vegetable crisper such as that used in most home refrigerators is useful for this purpose.

D. Tight-fitting plastic petri dishes may be used in an incubator that is not capable of maintaining the necessary high humidity level. In such containers, required humidity conditions are established with evaporation of some of the culture medium within the confines of the container (the petri dish).

Item 59. **Counting**

A. The light source must be adjusted for proper angle of illumination to the surface of the membrane for optimum observation of colonies. High angle (60 to 80 degrees) is necessary for counts on differential media. This is particularly critical in coliform differentiation based on the development of a golden metallic luster.

B. The procedure of drying membrane filter cultures prior to counting concentrates the sheen luster and may give a better color contrast to individual technicians who prefer this additional operation. However, comparisons made in several laboratories and by different technicians indicate no significant advantage for this membrane drying procedure. Using the improper lighting angle for coliform differentiation may have led some technicians into adopting this approach to colony counting.

C. The typical coliform colony has a pink to dark red color with a metallic surface sheen. The sheen area may vary from a small pin-head size to complete coverage of the colony surface. All members of the coliform group grow and develop a metallic sheen on this medium (4: page 613).

Non-coliform colonies vary from colorless to a deep red color,

but a sheen is never present. Colonies with a red color and a "small flake" or "speck" of shiny material resembling a metallic sheen are the most confusing of the non-coliform types. The novice has the greatest difficulty with confluent colonies and mirror reflections of fluorescent tubes, which are confused with sheen. Water condensate and turbidity particles are at times mistaken for colonies. Thus, there is a tendency for the novice to err on the high side in membrane filter counts. Inexperienced technicians who have not attained proficiency in coliform colony classification should transfer doubtful colonies to lactose (or lauryl tryptose) broth tubes for verification as coliform organisms.

D. Ideal membrane filters should have less than 60 coliform colonies. Exceeding the limit of 80 coliform colonies will result in lower coliform counts because of suppression of sheen or a spreading growth over the membrane with few distinct colonies (5.1: page 614).

Development of colonies from non-coliform flora is generally restricted to a great extent by the medium, but there are exceptions. A number of non-coliform colonies will always be present on the membrane filter except in samples such as sewage in which the coliform density numerically exceeds the non-coliform bacteria by many times.

E. Coliform densities counted by the membrane filter procedure are always reported as "coliform per 100 ml." This membrane filter coliform value can be calculated from one or more membrane filter counts in the 20- to 60-colony range. If the filter counts are less than 20, all counts should be totalled and the value based on the total volume of sample filtered (6: page 615).

F. If there are excessive colonies on the membrane, they should be reported as "TNC" (Too Numerous to Count), or if the growth has no well-defined colonies, it should be reported as "confluent". A new sample shall be requested, and filtration volumes reported.

Item 60. Number of tests per year

The laboratory or engineering section should have available a summary of the number of membrane filter positive and negative results for examination of public water supplies during the past 12 months.

LABORATORY SAFETY

Item 61. **Recommended laboratory practices**

A. The laboratory should be located in a clean, well-lighted, well-ventilated room. A light intensity of 50 foot-candles is recommended at all working surfaces.

B. The laboratory quarters should have reasonable freedom from dust, drafts, and excessive temperatures.

C. The water laboratory should be separated from the diagnostic laboratory as a safety precaution. Common manipulations in the laboratory such as use of pipets, inoculating needles, centrifuge, and blendors create aerosols, as do accidents such as dropping a petri dish or test tube. These accidents have created aerosols that have caused infections in persons stationed some distance away from the point of origin in the laboratory.

D. The laboratory quarters should have adequate cabinets, drawers, and/or shelves for protection of glassware, apparatus, and other materials as needed, especially when sterilized equipment is not used immediately after removal from sterilization ovens or autoclaves.

E. The laboratory table or bench should be level, with ample surface available for processing new samples, transferring positive presumptive tubes, and/or filtering samples by the membrane filter procedure. If the area used for filtering water samples through the membrane filter is not level, colony distribution may not be uniformly spread over the effective filter surface.

F. Floors of the laboratory should be clean and free from clutter and projections that might trip personnel. Floors should be kept dry, and if waxed, a non-skid wax should be chosen.

G. Electrical circuits should comply with local electrical codes and be properly grounded. Electrical grounding of water baths is a particularly important safety precaution.

H. Autoclaves should have periodic inspection by a representative of the manufacturer for preventive maintenance. Operating instructions for autoclaves and stills should be posted nearby, particularly if such equipment may be used by personnel on weekends or holidays when those routinely responsible for operations are away.

I. Each laboratory should have a copy of a manual on labora-

tory safety (such as that available from the Fisher Scientific Company) and a laboratory emergency treatment chart for providing guidelines in first-aid treatment of accident victims. First-aid cabinet contents should be checked frequently to replace out-of-stock items or items that have limited shelf lives.

SUPPLEMENTARY BACTERIOLOGICAL METHODS

STANDARD PLATE COUNT

Item 62. **Sample agitation**

A. It is necessary to shake the sample vigorously 25 times according to the provisions in Standard Methods to ensure proper distribution within the water sample (1:page 592).

B. It is permissible to use the dilution bottle mechanical shaker to ensure uniform distribution of bacteria within water samples.

C. It is recommended that sterile, buffered, distilled water be used for dilution water since all other diluting fluids are prohibited by the provisions of Standard Methods (1:page 592).

Item 63. **Plating**

A. Standard plate count should not be more than 300 colonies per plate. To obtain colony counts at or above 300 colonies, a decimal fraction of a milliliter of water sample should be plated (4:page 593).

B. Not less than 10 ml of melted medium should be added per plate (2:page 592).

C. Pouring the medium with temperature above 45°C may kill some viable bacteria, resulting in an underestimation of the colony count. It is recommended that the medium be tempered in a water bath at 43° to 45°C until medium reaches this temperature before the plate is poured (2:page 592).

D. No more than 20 minutes should elapse between making the dilution and pouring the plate, because on standing, bacterial multiplication may occur in the dilution bottle or fluid on the plate, resulting in erroneous colony counts.

Item 64. **Incubation**

A. Plates incubated at 35° ± 0.5°C should be counted after 24 (± 2) hours (3:page 592).

B. Plates incubated at 20° ± 0.5°C should be counted after 48 (± 3) hours rather than after 24 hours (3:page 592).

C. Plates with glass tops should be inverted during incubation to prevent water of condensation forming and dropping on the agar surface (3:page 592).

Item 65. **Counting**

A. Plates having more than 300 colonies per plate do not yield statistically valid data. It is necessary to use the serial dilution technique to plant a smaller sample of water (4:page 593).

B. To avoid fictitious accuracy and express results consistent with precision of the counting method, only the first two significant digits and "0" in the remainder of the whole number should be recorded when the count consists of 3 or more digits. The second digit should be increased by 1 when the third digit is 5 or greater, but do not change second digit when third digit is less than 5. For example, 146 is reported as 150, but 144 would be reported as 140 (4:page 593).

DIFFERENTIAL PROCEDURES

Item 66. **Elevated-temperature tests**

A. All positive presumptive tubes (lactose broth or lauryl tryptose broth) are confirmed in EC medium and incubated in a carefully regulated water bath at 44.5° ± 0.5°C for 24 hours. Gas production in the fermentation tube within 24 hours or less is considered a positive reaction, indicating fecal origin of the organisms (5:page 599).

B. Boric acid lactose broth may be used in an alternative procedure, but the water bath incubation temperature must be 43° ± 0.5°C for 24 to 48 hours. Gas production within 48 hours or less is considered a positive reaction.

C. All inoculated tubes of either EC medium or boric acid lactose broth must be placed in the water bath within 30 minutes (5.1a:page 600).

D. Air incubation is not permitted for the elevated-temperature test since incubation temperature is the critical differential mechanism. Since the specificity of the fecal coliform test is directly related to the incubation temperature, there is a need for a greater temperature control of the water bath. Temperature tolerance of ± 0.2°C can be obtained with several types of water baths (approximately 36- by 18-inch chamber size) available on the commercial market.

Item 67. **Differential tests (tentative procedures)**

A. Further classification of coliform strains may be made by a study of their biochemical reactions to the IMViC series of tests.

B. These tests are applied to pure cultures of coliform strains, such as those recovered from the completed test.

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APPENDIX I

EVALUATING THE ACCURACY OF MULTIPLE-TUBE TESTS

Frequently, stream pollution investigations are carried out in the same laboratory used for bacterial examination of water samples. For such studies, five portions each in three usable dilutions are recommended as the minimum sample to yield acceptable data. The following table (Table I) is useful in evaluating the accuracy of such multiple-tube tests when work sheets are available. Table I gives the typical positive tube combination, MPN, the 95 percent confidence ranges, and the logarithm of the MPN. Indeterminate results in which all portions were either negative (0-0-0) or positive (5-5-5) are omitted.

It is further divided into groups: Class 1 group should include 67.5 percent of all tests, and when Class 2 group is added to Class 1 group, the two should include 91.1 percent of the samples examined. Such an analysis should be based on a minimum of 50 MPN combinations made available from laboratory work sheets.

If more than 8.9 percent of the MPN combinations examined are not included in the above two classes, the multiple-tube procedure is probably in error. Such an abnormal distribution might result from substances in the water that inhibit bacterial growth, from improper laboratory procedures, or other causes. Certainly it indicates the desirability of special investigations to explain lack of consistency with the normal pattern.

Such employees can carry out or supervise routine test procedures, training activities, consultations on methods and problems, and evaluations of new or routine procedures as needed. In view of the great increase in the number of samples collected during the summer months, temporary help with qualifications acceptable to, and under direct supervision of the bacteriologists, can be added as required.

If followed, the two recommendations below increase the efficiency of laboratory operations and result in over-all economies:

1. Media kitchens, and dish washing services should be consolidated so they can prepare all media required for the water laboratory, for the milk laboratory, and probably for other laboratory services. This would result in economies in operation, less personnel time assigned to these duties, and less duplication of equipment needed for such services (autoclaves, hot-air sterilizers, etc.).
2. Laboratory space should be relocated so that all laboratory services are on a single floor. Those laboratories engaged in similar work should be close together so they can share common equipment, such as incubators, etc., or specialized apparatus that are used only occasionally.

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE

Survey Form for Water Laboratories (Bacteriological)

PHS-875
(Rev. 1-66)

Indicating conformity with Public Health Service Drinking Water Standards

SURVEY BY

X=DEVIATION U=UNDETERMINED 0=NOT USED

LABORATORY

LOCATION

DATE

SAMPLES

1. Sampling ____
- Representative points throughout distribution system ____
- Location and frequency of sampling adequate ____
- Minimum number monthly adequate for population ____
- Repetitive samples from designated points as well as others to establish bacteriological quality throughout system ____
- Proportionately more samples from small population areas ____

2. Collection Procedure ____
- Volume necessary for all tests ____
- Minimum sample not less than 100 ml ____
- Ample air space shall be left in bottle to facilitate mixing ____
- Representative sample collected without contamination ____
- Tap sample collected from tap connected directly to main by service pipe ____
- Do not collect sample from tap connected to storage tank ____
- Tap allowed to waste water until service line has been cleared ____
- River, stream, lake, or reservoir sample collected by plunging opened bottle with neck downward below surface ____
- Collect with mouth end toward current (or away from hand) ____
- Pump allowed to waste water 5 min before taking sample ____
- Promptly identify sample legibly and indelibly ____
- Complete and accurate data accompanies sample ____

3. Dechlorination ____
- Sodium thiosulfate used for dechlorination ____
- Added before sterilization of bottle ____
- Concentration approximately 100 mg/l ____

4. Transportation and Storage ____
- Temperature maintained as close as possible to the water temperature at time of collection ____
- Initiate examination as soon as possible after collection ____
- Recommended time within 1 hr after collection ____
- Time between collection and examination not exceeding 30 hr ____
- Establish field procedure where time exceeds 24 hr ____
- Time and temperature of storage recorded ____

5. Remedial Action For Unsatisfactory Sample ____
- If three or more positive tubes per test, or ____
- Four or more coliforms per 100 ml by membrane filter ____
- Daily samples from point promptly examined until two consecutive samples are satisfactory ____

6. Record of Laboratory Examinations ____
- Results assembled and available for inspection ____
- Consistent compliance with water quality standards ____
- Laboratory methods and technical competence of local government, waterworks, and commercial laboratories approved by reporting agency ____

LABORATORY APPARATUS

7. Sample Bottles ____
- Glass resistant to solvent action of waters ____
- Plastic sample bottles which may be sterilized and yield no toxic substances ____
- Holds sufficient sample with air space for all necessary bacteriological tests, maintains sample uncontaminated ____
- Closure: ____
  - a. Glass stopper covered, before sterilization, with metal foil, rubberized cloth, or heavy impermeable paper ____
  - b. Metal or plastic screw cap with leak-proof liner, provided both are free from toxic substances on sterilization ____

8. Thermometer ____
- Accuracy checked with thermometer certified by National Bureau of Standards, or one of equivalent accuracy ____

9. Incubator ____
- Maintain uniform temperature in all parts (±1.0° C) ____
- Either water-jacket (filled) or anhydric type, with low-temperature, thermostatically controlled electric heating units properly insulated and located in or adjacent to walls or floor ____
- Provided with shelves so spaced as to assure uniformity of temperature ____
- Sufficient size (provide 1-in. space between walls, dishes, or baskets) ____
- Accurate thermometer with bulb immersed in liquid on each shelf ____
- Daily record of temperature, or ____
- Optionally use automatic devices of predetermined accuracy for recording temperatures ____
- Unless recording thermometers are in continuous operation, preferably install maximal and minimal registering thermometer on middle shelf to record temperature variations over 24-hr period ____
- At intervals determine temperature variations within incubator when filled to maximal capacity ____
- Keep where temperatures do not vary excessively (50-80°F) ____

10. Incubator Room ____
- Optionally use walk-in rooms, well insulated, equipped with properly distributed heating units and forced-air circulation ____
- Provided areas conform to desired temperature limits ____
- Record daily range in temperature in areas used for plates ____

11. Hot-Air Sterilizing Oven ____
- Size sufficient to prevent crowding of interior ____
- Constructed to give uniform and adequate sterilizing temperatures (check temperature variations within oven) ____
- Equipped with thermometer registering accurately at 160-180° C ____
- Recording thermometer (optional) ____

12. Autoclaves ____
- Size sufficient to prevent crowding of interior ____
- Constructed to provide uniform temperatures up to and including 121° C ____
- Equipped with accurate thermometer with bulb properly located to register minimal temperature within chamber ____

Laboratory Apparatus (continued)

Recording thermometer (optional) ____
Pressure gages and properly adjusted safety valve ____
Connected with saturated-steam line, or to gas or electrically heated steam generator ____
Reach sterilizing temperature in 30 min ____
Small pressure cookers may be substituted only in emergencies and only where satisfactory results have been demonstrated ____
And provided pressure cooker has pressure gage and thermometer with bulb 1 in. above water level ____

13. Colony Counter ____
Quebec colony counter, dark-field model preferred ____
Or one providing equivalent magnification and visibility ____

14. pH Equipment ____
Electrometric pH meter shall be used for pH of media ____

15. Balances ____
a. Balance with sensitivity of 2 g at 150-g load shall be used ____
Appropriate weights of good quality ____
b. Analytical balance with sensitivity of 1 mg at 10-g load ____
Appropriate analytical grade weights ____
Used for weighing quantities less than 2 g ____

16. Media Preparation Utensils ____
Borosilicate glass ____
Stainless steel ____
Other noncorrosive utensil ____
Clean and free from foreign residues or dried agar ____
Free from toxic or foreign materials which may contaminate media (such as detergents, chlorine, copper, zinc, antimony, or chromium) ____

17. Pipets ____
Deliver accurately and quickly ____
Calibration error not exceeding 2.5% ____
Tips unbroken, graduation distinctly marked ____
Mouth end plugged with cotton (optional) ____
Pipets conforming to APHA specifications (S. M. Exam. Dairy Prod. 11th ed.) may be used (optional) ____

18. Pipet Containers ____
Box, aluminum or stainless steel, 2-3 x 16 in. ____
Paper wrappings of good quality sulfite paper (optional) ____
Copper cans or boxes prohibited ____

19. Dilution Bottles or Tubes ____
Borosilicate or other noncorrosive glass ____
Glass stopper or ____
Screw cap with leak-proof liner free from toxic substances on sterilization ____
Cotton plugs prohibited ____
Graduation level indelibly marked on side of bottle or tube ____
Non-toxic plastic bottles may be substituted ____

20. Petri Dishes ____
Clear bottom, free from bubbles and scratches ____
Diameter 100 mm x 15 mm high (60 x 15 mm for M.F.) ____
Bottom flat for medium of uniform thickness ____
Glass or porous top ____
Presterilized plastic dishes proven free from toxic substances and sterile may be substituted for single use only ____

21. Petri Dish Containers ____
Used to protect and handle before and after sterilization ____
Aluminum or stainless steel (not copper) cans with covers, coarsely woven wire baskets or char-resistant paper sacks or wrappings ____

22. Fermentation Tubes ____
Sufficient size to conform with requirements for concentration of nutrient ingredients and sample as described subsequently ____

PREPARATION

23. Cleaning Glassware ____
Thoroughly washed with suitable detergent and hot water (160° F) ____
Rinsed in clean water at 180 °F to remove detergent ____
Rinsed with distilled water ____
Freedom from any residue on drying ____
Free from acidity or alkalinity ____
Detergent leaves no toxic residue ____

24. Sterilization of Glassware ____
Heat glassware not in metal containers for not less than 60 min at 170° C ____
Optionally use 160° C for 60 min with constant temperature recording device if oven temperatures are uniform ____
Heat glassware in metal containers for not less than 2 hr at 170° C ____
Non-plastic sample bottles as above, or ____
Autoclave at 121° C for 15 min ____
Plastic sample bottles that distort on autoclaving sterilized by low-temperature ethylene oxide gas ____

25. Buffered Dilution Water ____
Stock phosphate buffer solution at pH 7.2 ____
Freshly prepared when stock solution shows trace of turbidity ____
Optionally autoclave stock buffer and store in refrigerator ____
1.25 ml of stock buffer added to 1 liter distilled water ____
Dispense to give 99± 2 ml or 9± 0.2 ml after autoclaving ____
Sterilized in autoclave at 121° C for 15 min ____
Quantity after sterilization with 2% or less deviation ____
Dilution volumes of 9 ml may be measured aseptically ____

MEDIA — MATERIALS AND PROCEDURES

26. Water ____
Distilled or demineralized water used for media, reagents, blanks, etc. ____
Free from traces of dissolved metals or chlorine ____
Neutralize distilled water if free chlorine is present ____
Free from bactericidal compounds as measured by bacteriological suitability test using *Aerobacter aerogenes* ____

27. Media Ingredients ____
Beef extract ____
Yeast extract ____
Peptone ____
Sugars ____
Agar ____
Above preparations demonstrated to give satisfactory results for bacteriological purposes ____

28. General Chemicals ____
Reagent or ACS grade ____

29. Dyes ____
Certified by the Biological Stain Commission for use in media ____

30. Storage ____
Dehydrated media stored tightly in dark (low humidity) at less than 30° C ____
Not used if discolored or caked ____
Culture media stored in clean, dry space free from contamination and excessive evaporation ____

Survey Form for Water Laboratories (Bacteriological)

X=DEVIATION U=UNDETERMINED 0=NOT USED

LABORATORY	LOCATION	DATE

Media — Materials and Procedures (continued)

Batches used in less than 1 week ____
Protected from strong light ____
Fermentation tubes stored at room temperature not over 1 week ____
Fermentation tubes stored at low temperatures must be incubated overnight and tubes with air bubbles discarded ____

31. Adjustment of Reaction ____
Expressed in concentration of hydrogen ions (pH) ____
Determine decrease in pH of individual medium in the autoclave used for its sterilization ____
Potentiometric method recommended for accurate determination ____
Appropriate standard buffer used to calibrate pH meters ____
Colorimetric method not used with media containing dyes ____
Indicator solution prepared to match color standards ____
Calibrated against standard buffer ____
Follow standard procedure, using a comparator ____

32. Sterilization of Media ____
All media, except sugar broths, autoclaved at 121° C for 15 min ____
Timing starts when autoclave reaches 121° C temperature ____
Media removed and cooled as soon as possible after sterilization ____
Tubes packed loosely in baskets for uniform heating and cooling ____
Carbohydrate broths sterilized as above for 10 min ____
Optionally add sterilized carbohydrate solution aseptically to sterile media ____
Incubate tubes so prepared at 35° C for 24 hr before use ____
Total exposure of carbohydrate media to heat not over 60 min ____

33. Clarification ____
Use filtration, sedimentation, or centrifugation as needed ____
Do not clarify with nutrient substances such as egg albumen ____

CULTURE MEDIA — SPECIFICATIONS

34. Per Cent Solution ____
Use grams solute per 100 ml of solution ____

35. Nutrient Broth ____
Correct composition, sterility, and pH (6.8-7.0) ____

36. Lactose Broth ____
Correct composition, sterility, and pH (6.9±0.1) ____
Tubed in proper sized tube with inverted vial ____
Total time of exposure to any heat not more than 30 min ____
When quantities greater than 1 ml are planted, composition after planting will contain 0.013 g per ml of dry ingredients ____
Not less than 10 ml medium per tube ____
Dehydrated medium used. Brand ..... Lot No. ____

37. Lauryl Tryptose Broth ____
Correct composition, sterility, and pH (6.8 ± 0.1) ____
Complies with general requirements described above ____
When quantities greater than 1 ml are planted, composition after planting will contain 0.0356 g per ml of dry ingredients ____
Dehydrated medium used. Brand ..... Lot No. ____

38. Brilliant Green Lactose Bile Broth ____
Correct composition. Brand ..... Lot No. ____
Dehydrated ox gall only used in medium ____
Ox gall solution (10% in distilled water) has pH of 7.0 to 7.5 ____
Add 13.3 ml 0.1% solution brilliant green/l of medium ____
Sterile medium has pH of 7.1 to 7.4 (electrometric method only) ____

39. Endo Medium ____
Formula I used or ____
Formula II used ____
Correct composition, sterility, and pH (7.4 ± 0.1) ____
Proper reaction when seeded with coliforms ____
Dehydrated medium used. Brand ..... Lot No. ____

40. Eosin Methylene Blue Agar ____
Correct composition, sterility, and pH (7.1 ± 0.1) ____
Proper reaction when seeded with coliforms ____
Medium contains no sucrose. Catalog No. ____
Dehydrated medium used. Brand ..... Lot No. ____

41. Tryptone Glucose Beef Extract Agar ____
Tryptone Glucose Yeast Extract Agar ____
Correct composition, productivity, sterility, and pH (7.0 ± 0.1) ____
Sterile medium not remelted a second time after sterilization ____
Free from precipitate ____
Dehydrated medium used. Brand ..... Lot No. ____

42. M-Endo Medium ____
Correct composition. Brand ..... Lot No. ____
Prepare in clean presterilized borosilicate glassware ____
Reconstituted in distilled water containing 2% ethyl alcohol ____
Heated to boiling point (do not boil or submit to steam pressure) ____
pH between 7.1 and 7.3 ____
Stored in dark at 2-10° C ____
Unused medium discarded after 96 hr ____

43. M-Endo Agar Medium ____
Correct composition. Brand ..... Lot No. ____
Reconstituted in distilled water containing 2% ethyl alcohol ____
Heated to boiling point (do not boil or submit to steam pressure) ____
pH between 7.1 and 7.3 ____
Cool to 45-50° C and dispense 4 ml in 60-mm dishes ____
Keep plates in dark at 2-10° C (may store 2 weeks) ____

TESTS FOR PRESENCE OF MEMBERS OF COLIFORM GROUP BY MULTIPLE-TUBE FERMENTATION TECHNIQUE

44. Presumptive Test ____
Lactose broth or ____
Lauryl tryptose broth ____
Before planting portions arrange tubes in order, number sample, or otherwise identify ____
Shake sample vigorously 25 times before removing portions ____
Inoculate fermentation tubes with appropriate quantities ____
Use 5 standard portions, either 10 or 100 ml ____
Concentration of ingredients conform to items 36 or 37 ____
Quantities inoculated: 10 ml ..... 1 ml ..... 0.1 ml ____
Incubate tubes at 35 ± 0.5° C for 24 ± 2 hr ____
Examine for gas — any gas bubble positive ____
Return negative tubes to incubator ____
Examine for gas at 48 ± 3 hr from original incubation ____
Record presence or absence of gas at each examination ____
Gas in any quantity in 48 ± 3 hr is positive Presumptive Test ____
No gas in 48 ± 3 hr is negative test ____
Do not record gas produced after 51 hr of incubation ____

Tests for Presence of Members of Coliform Group by Multiple-tube Fermentation Technique (continued)

45. Confirmed Test .....

Promptly submit all presumptive tubes showing gas production before or at 24-hr and 48-hr periods to Confirmed Test .....

a. Brilliant green lactose bile broth .....

Gently shake tube or mix by rotating .....

Transfer one loopful of positive broth to brilliant green bile.....

Using sterile loop of 24-gage wire not less than 3 mm diameter.....

Incubate for 48 ± 3 hr at 35° ± 0.5° C .....

Formation of any gas in the inverted vial within 48 ± 3 hr constitutes a positive Confirmed Test .....

b. Endo or eosin methylene blue agar plates .....

Streak one or more plates with inoculum from positive primary fermentation tube(s) .....

Use inoculating needle slightly curved at tip .....

Tap and incline tube to avoid any scum .....

Insert needle approximately 5 mm .....

Streak agar surface with curved section .....

To obtain discrete colonies separated by at least 0.5 cm .....

Incubate at 35° ± 0.5° C for 24 ± 2 hr .....

If typical nucleated colonies with or without sheen develop, the Confirmed Test is positive .....

If atypical, unnucleated pink colonies develop, result is doubtful and Completed Test must be applied .....

If no colonies or only colorless colonies without mucoid characteristics, the Confirmed Test is negative .....

c. Alternate application with multiple portions and dilutions .....

Optional only with three or more decimal dilutions .....

Submit to Confirmed Test all positive tubes of two highest dilutions (smallest volumes) occurring in 24 hr .....

Record any tube showing gas in 24 hr which has not been confirmed as positive Confirmed Test .....

Submit to confirmation all tubes negative at 24 hr and positive in 48 hr (no exception) .....

46. Completed Test .....

Applied to positive Confirmed Test fermentation tubes or .....

To doubtful appearing colonies on differential plate medium .....

If from liquid medium, streak as in item 45b .....

Fish one or more typical colonies to transfer to lactose or lauryl tryptose broth tube and to an agar slant or .....

Fish two or more atypical colonies as described above .....

Choose colonies separated by 0.5 cm .....

Incubate at 35° ± 0.5° C for 24 ± 2 or 48 ± 3 hr.....

Examine agar slant with Gram-stain if corresponding secondary lactose broth showed gas in 24 or 48 hr .....

Gram-negative rods without spores and gas in lactose tube is positive Completed Test .....

Absence of gas in lactose tube in 48 hr — negative test .....

Only Gram-positive bacteria in stain — negative test .....

47. Application of Tests to Routine Examination .....

a. Presumptive Test applied to: .....

Samples not being considered for drinking water .....

Routine raw water quality where applicable in plant .....

b. Confirmed Test applied to: .....

Waters to which Presumptive Test is not applicable .....

Routine samples of drinking water, process or finished water....

Chlorinated sewage effluents .....

Bathing waters .....

c. Completed Test applied to: .....

Examination of water samples where results are to be used for the control of the quality of raw or of finished waters .....

If not applied to all such samples, then applied to such a proportion as to establish beyond reasonable doubt the value of the Confirmed Test in determining their sanitary quality..

48. Number of Tests Per Year .....

Presumptive Total ..... + ... — .....

Confirmed Total ..... + ... — .....

Completed Total ..... + ... — .....

TESTS FOR PRESENCE OF MEMBERS OF COLIFORM GROUP BY MEMBRANE FILTER TECHNIQUE

49. Application As Standard Test .....

Use as a standard test for determining potability of water after demonstration by parallel testing that it yields information equal to that from the multiple-tube fermentation procedure.....

Examine not less than 50-ml sample .....

50. Laboratory Apparatus (See items 7, 17-21) .....

Graduated cylinders accurate within 2.5% and .....

Openings covered with metal foil or substitute before sterilization

Use glass containers for culture medium .....

Filtration unit — any type that can be sterilized .....

Filters all the sample through the membrane .....

Vacuum source (water trap to protect pump desirable) .....

Funnel and filter receptacle wrapped separately for sterilization and storage .....

51. Filter Membranes .....

Full bacterial retention, satisfactory speed of filtration .....

Stable in use, glycerin free, free from substances toxic to growth

Preferably grid marked, non-toxic ink .....

Adequately protected during sterilization from recontamination....

Autoclaved at 121° C for 10 min (or presterilized) .....

52. Absorbent Pads .....

Filter paper free from growth inhibitory substances .....

Approximately 48 mm diameter .....

Thickness will permit absorbing 1.8 - 2.2 ml medium .....

Sterilized before using in test (121° C for 10 min) .....

53. Forceps .....

Round tipped, without corrugations .....

Membranes and pads handled with sterile (alcohol flamed) forceps .....

54. Microscope and Lamp .....

Preferably binocular wide field, 10 to 15 diameters magnification

Fluorescent light, adjacent, above, perpendicular to filter plane....

Other optical device giving equivalent results .....

55. Filtration .....

Suitably disinfect bench surface, allow to dry .....

Filter holding unit sterile at start of series .....

Use support for inverted funnel between samples (optional) .....

Place sterile membrane on porous plate, secure funnel .....

Apply vacuum, filter appropriate sample volume (number of tests uncountable not excessive) and rinse funnel .....

By filtering 3 volumes of 20-30 ml of sterile buffered water .....

Remove filter with sterile forceps .....

56. Standard Test — Single Step .....

a. Broth— Sterile pad placed in culture dish .....

Saturated with M-Endo medium (42) .....

Allow to stand a few minutes before pouring off excess

Prepared filter (55) rolled (grid side up) onto pad....

b. Agar— Use culture dish previously prepared (43) .....

Prepared filter (55) placed on agar with rolling motion .....

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Survey Form for Water Laboratories (Bacteriological)

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LABORATORY	LOCATION	DATE

Tests for Presence of Members of Coliform Group by Membrane Filter Technique (continued)

57. Standard Test — with Enrichment ____
- Sterile pad placed in culture dish ____
- Saturated with approximately 1.8 ml enrichment medium (37) ____
- Carefully remove any excess liquid ____
- Prepared filter (55) placed on pad ____
- Incubate inverted filter at 35° ± 0.5° C for 1½-2 hr ____
- Remove dish from incubator and remove top ____
- **a. Broth** — With sterile forceps lift pad and filter to top of dish ____
  - Place new sterile pad on bottom and saturate with medium (42) ____
  - Transfer filter to new pad (and discard used pad) ____
- **b. Agar** — With sterile forceps strip filter from pad ____
  - Roll onto surface of previously prepared agar (43) ____
  - Optionally leave used pad in dish to maintain humidity ____

58. Incubation ____
- In saturated humidity, with dish inverted ____
- At 35° ± 0.5° C, for 22-24 hr ____
- Incubate enriched tests for 20-22 hr ____

59. Counting ____
- Count all dark colonies with sheen as coliform colonies ____
- If only atypical forms, establish identity ____
- Calculation of direct count in density per 100 ml ____

60. Number of Tests Per Year ____
- Membrane Filter Total ..... + ... — ..... ____

MISCELLANEOUS

61. Recommended Laboratory Practices ____
- Personnel adequately trained or sup[illegible] ____
- Copy of Standard Methods (12th ed. [illegible]ble in laboratory ____
- Level table or bench, ample working [illegible] ____
- In clean, well-lighted, well-ventilated room, reasonably free from dust and drafts ____
- Floors clean, walls and ceilings smooth ____
- Doors and windows screened, or insects and rodents absent ____
- Space adequate, free from confusion ____
- Used for laboratory purposes only ____
- Table space, storage, and utilities adequate ____
- Cabinets, shelves, and equipment neat, clean, and orderly ____
- Clean outer garments worn ____
- Clothing stored outside laboratory or in closet ____
- Safety precautions and practices satisfactory ____

SUPPLEMENTARY BACTERIOLOGICAL METHODS

Applicable to non-potable surface, process and bathing waters, effluents, sea water, and shellfish.

STANDARD PLATE COUNT

62. Sample Agitation ____
- Shaken vigorously 25 times immediately before removing portion ____
- Each dilution shaken 25 times ____

63. Plating ____
- Appropriate quantity of sample per plate ____
- In highly polluted waters use dilutions in sterile dilution water ____
- Plate not more than 1 or less than 0.1 ml (sample or dilution) ____
- Add to each dish 10 ml or more liquefied agar medium ____
- At temperature of 43-45° C ____
- Melted medium stored for no more than 3 hr, 43-45° C ____
- Gently lift cover of petri dish only high enough to pour medium ____
- Agar and test portions thoroughly mixed, by rotating and tilting without splashing, and mixture spread evenly ____
- Allow to solidify within 5-10 min on level surface ____
- Invert (unless clay tops are used) and promptly incubate ____
- Not more than 20 min between measuring sample and pouring ____

64. Incubation ____
- Arrange so each plate or pile is separated by at least 1 in. from adjacent piles, and from top and walls of chamber ____
- Incubate at 35° ± 0.5° C for 24 ± 2 hr, or ____
- At 20° ± 0.5° C for 48 ± 3 hr ____
- Any deviation stated in report ____
- For shellfish incubate 48 ± 3 hr at 35° C ____

65. Counting ____
- Record count observed on 1 ml sample with less than 30 colonies ____
- Otherwise count only plates with between 30 and 300 colonies ____
- Count colonies with approved counting aids ____
- Record only two significant figures ____
- Raise second digit from left by 1 when third digit is 5 or more ____
- Record as "standard plate count at 35° C (or 20° C)" ____

DIFFERENTIAL PROCEDURES

66. Elevated Temperature Tests ____
- Applied to positive presumptive or coliform colony ____
- Inoculate fermentation tube of EC medium (3 mm loop) ____
- Placed in water bath within 30 min ____
- Hold at 44.5° ± 0.5° C for 24 hr ____
- Gas production is positive test (indicating fecal origin) ____
- Absence of gas is negative reaction (non-fecal origin) ____
- Used only as confirmatory test ____

67. Differential Tests (Tentative) ____
- **a. Indole tests** ____
  - Tryptophane Broth (35° C, 24 ± 2 hr) ____
  - Satisfactory reagent (pH < 6.0) ____
  - Procedure satisfactory ____
- **b. Methyl red test** ____
  - Buffered glucose broth (35° C, 5 days) ____
  - Satisfactory indicator ____
  - Procedure satisfactory ____
- **c. Voges-Proskauer test** ____
  - Buffered or salt peptone glucose broth (35° C, 48 hr) ____
  - α-napthol solution fresh daily ____
  - Procedure satisfactory ____
- **d. Sodium citrate test** ____
  - Koser's citrate broth (35° C, 3-4 days) or ____
  - Simmon's citrate agar (35° C, 48 hr) ____
  - Procedure satisfactory ____

TABLE I

STATISTICAL EXPECTANCY OF MPN POSITIVE TUBE COMBINATIONS

Class 1. MPN tube combinations in 67.5% or more samples

Sample size			95% Confidence zone			
10 ml	1 ml	0.1 ml	MPN	Low	High	log MPN
1	0	0	2	< 0.5	7	0.30103
2	0	0	5	< 0.5	13	0.69897
3	0	0	8	1	19	0.90309
4	0	0	13	3	31	1.11394
5	0	0	23	7	70	1.36173
5	1	0	33	11	93	1.51851
5	2	0	49	17	130	1.69020
5	3	0	79	25	190	1.89763
5	4	0	130	35	300	2.11394
5	5	0	240	68	750	2.38021
5	5	1	348	120	1000	2.54158
5	5	2	542	180	1400	2.73400
5	5	3	918	300	3200	2.96379
5	5	4	1609	640	5800	3.20412

Class 2. MPN tube combinations in 23.6% or more samples

Sample size			95% Confidence zone			
10 ml	1 ml	0.1 ml	MPN	Low	High	log MPN
1	1	0	4	< 0.5	11	0.60206
2	1	0	7	1	17	0.84510
3	1	0	11	2	25	1.04139
4	1	0	17	5	46	1.23045
4	2	0	22	7	67	1.34242
5	1	1	46	16	120	1.66276
5	2	1	70	23	170	1.84510
5	3	1	109	31	250	2.03743
5	4	1	172	43	490	2.23553
5	4	2	221	57	700	2.34439

APPENDIX II

RECOMMENDATIONS FOR REORGANIZATION OF LABORATORY SERVICES

Bacteriological services in the sanitation laboratory may include the examination of potable waters, untreated waters from surface sources or ground supplies, stream pollution investigations, fresh or saline waters from bathing areas, surveillance of swimming pools, salt waters from shellfish areas, and the bacteriological examination of shellfish. Official methods are prescribed in various "Standard Methods," for all these services except examination of bathing areas, swimming pools, and stream pollution studies. Where official methods are not prescribed, the procedures should follow good and accepted laboratory procedures and, when applicable, be in compliance with local statutes or administrative regulations. Where needed the laboratory services should be expanded to include a training program of 1 or 2 weeks for all new employees in laboratories (municipal, water plants, private) or refresher courses (as needed) for employed personnel who perform bacteriological examinations of water samples. This program should include visits to and consultation on methods of the laboratories and an eventual approval system (on a voluntary basis or by administrative regulation or by statute) for all laboratories from which data are accepted for control or regulatory action.

Ideally bacteriologists for this program should include two permanent employees with the following qualifications:

1. Senior Bacteriologist with a major in bacteriology from a recognized college and a MA (or MS) degree or equivalent experience in water bacteriology.
2. Bacteriologist with a major in bacteriology from a recognized college or equivalent experience in water bacteriology.